UNIVERSITY OF EDINBURGH.

PHYSICOCHEMICAL STUDIES ON STARCHES: THE DETERMINATION OF THE SIZE AND SHAPE OF THE COMPONENTS IN SOLUTION.

by

IAN G. JONES, B.Sc.

T H E S I S

submitted for the degree of

DOCTOR OF PHILOSOPHY.

October, 1959.

C O N T E N T S.

PREFACE.

Throughout the period of this research, Dr. C.T. Greenwood has been a constant source of advice and encouragement and I wish to take this opportunity to express my gratitude to him. Some of the work described in Sections III and IV has been published in conjunction with Dr. Greenwood and reprints of these papers are inserted at the end of this thesis.

I wish to thank Professor E.L. Hirst for the provision of laboratory facilities and equipment and the Rockefeller Foundation for a grant.

I also wish to thank Dr. J.M.G. Cowie for his collaboration in the work recorded in Section IV and Dr. W.A.J. Bryce for carrying out diffusion measurements.

The University Chemistry Department,
The King's Buildings,
EDINBURGH, 9.

GENERAL INTRODUCTION.

Although much work has been carried out on the components of starch, amylose and amylopectin, their molecular size and shape has not yet been precisely established. Such knowledge, apart from its intrinsic interest, would be of considerable aid in elucidating the internal structure of the starch granule which is, as yet, only imperfectly understood. The work of the author over the past three years has been devoted to an attempt to make progress in this direction.

The size and shape of the amylose molecule in solution has been studied by Bryce (1958) and Cowie (1958) using unfractionated samples. It was felt that further advances could be made only through the use of well fractionated material and therefore investigation was made of several methods of subfractionation. Since this work has not yet proved successful, it has not been possible to proceed with any fundamental hydrodynamic studies.

The problem of the size and shape of amylopectin is inherently a very difficult one because of the strong non-ideal behaviour associated with solutions of such a large, highly branched molecule. The similar polysaccharide, glycogen, behaves rather more ideally and, therefore, as a preliminary to studies on amylopectin, the physicochemical properties of glycogen have been examined. Studies on glycogen may be even more pertinent to the understanding of starch than was at first realised in view of certain recent suggestions by Erlander (1958) that a glycogen-like material is the natural precursor of both amylose and amylopectin. If this

should prove to be the case, a knowledge of the behaviour of glycogen may well be of considerable value in determining the actual mechanism of this process.

In the first section, an outline of some current ideas about starch-type polysaccharides is given. Following this, the principles and practice of the physical methods used in this work are discussed. In particular, the technique of light scattering is treated fully as this method had not been previously applied in these laboratories. Section III is devoted to studies on glycogen in which the effect of isolation procedure is examined, the nature of the highest molecular weight material discussed and the determination of the molecular shape in solution attempted. In section IV the action of the starch degrading enzyme, β - amylase, on glycogen is investigated. Various studies of the molecular size of amylopectin are made in section V and section VI describes the attempts made to subfractionate amylose.

SECTION I.

THE STRUCTURE OF STARCH-TYPE POLYSACCHARIDES.

The starch-type polysaccharides - amylose, amylopectin and glycogen - are very closely related chemically in that they all consist of anhydro-glucose residues linked essentially through 1α4 glycosidic bonds. They differ, however, in their degree of branching and as this controls physical properties such as solubility, stability in solution and hydrodynamic behaviour, a study of these properties by physicochemical methods should enable the finer points of structural difference to be elucidated.

Branching in Starch-type Polysaccharides.

(1) Amylose.

Amylose, the minor component of starch, has the simplest structure. Although amylose is regarded as an essentially unbranched structure, some lines of evidence introduce the possibility of branching. These include (a) periodate oxidation, (b) enzymic degradation studies and (c) hydrodynamic behaviour. The implications of this type of work are discussed below.

(a). Results of periodate oxidation studies (Potter and Hassid,(1951), suggest the presence, in some samples of amylose, of up to 3 non-reducing terminal groups per molecule. However, the validity of these results is doubtful, in view of the considerable error which is caused by the presence of contaminating amylopectin.

(b). Enzymic degradation experiments have also suggested the presence of branching. The enzyme, β-amylase, which is known to degrade amylose in a step-wise manner from the non-reducing end with the

liberation of maltose, does not completely hydrolyse some amyloses. In fact, amyloses from all sources, when isolated by methods involving complete dispersion of the starch granule, are converted to only 65 - 85% of maltose by purified β-amylase. Amyloses which are hydrolysed completely can, however, be obtained by extraction methods which remove only a fraction of the total amylose from the granule (Cowie and Greenwood, 1958). As the degrading action of β-amylase will be halted by the existence of a branch-point in the amylose chain, these results may be considered as evidence for branching. However, it has been shown recently (Banks et al, 1959) that the enzyme action is stopped by several other artificially introduced anomalies. These barriers could either be present in the native amylose or could be inadvertently introduced during isolation and therefore it is not necessary to postulate the presence of side-chains in amylose to account for the observed β-amylolysis limits.

(c). The difference in solution behaviour of amyloses isolated from different sources also could suggest branching. Goodison and Higginbotham (1950), from studies of well-defined fractions of sago, tapioca and maize amyloses, found quite different relations between intrinsic viscosity and molecular weight for the three samples. In addition, they found that, for fractions of the same DP from the three sources, the magnitude of the gel-rigidity of a concentrated solution differed markedly. While this would seem to be conclusive evidence for differences in the degree of branching, it is possible that these results could be explained by the presence of anomalous or modified units which could hinder free-rotation at some point

in the molecule. A further possibility is the presence of ester phosphate groups which have been shown to be present in small amounts in amylose (Banks and Greenwood, 1959). The mutual repulsion of these charged groups could affect the apparent stiffness of the chain. Thus, although the presence of branching in amylose is still a possibility, there is, as yet, no conclusive evidence.

(2). Amylopectin.

The major component of most starches, and the sole component of waxy starches, is amylopectin. Although this is still essentially a 1-α-4 linked glucose polymer, a fairly high degree of branching is present. Methylation and periodate studies both show that the main interchain link is through a 1-α-6 bond. The average degree of branching for most amylopectins appears to be 4 - 5% (Greenwood 1956). However, the possibility of the presence of other interchain bonds has been suggested by the isolation, from partial hydrolysates of waxy maize starch, of a small amount of the 1-α-3 disaccharide, nigerose (Wolfrom and Thomson, 1956). Abdel-Akher and Smith (1952) have found, in hydrolysates of periodate-oxidised amylopectin, some 0·5% of glucose, suggesting that some interchain linkages occur either C_2 or C_3. Neither of these results can be considered conclusive, but the possibility cannot be excluded that a small proportion of interchain bonding through C_2 or C_3 is present.

(3) Glycogen.

Glycogen, from both animal and plant sources, is rather more highly branched than amylopectin, but the main branching occurs

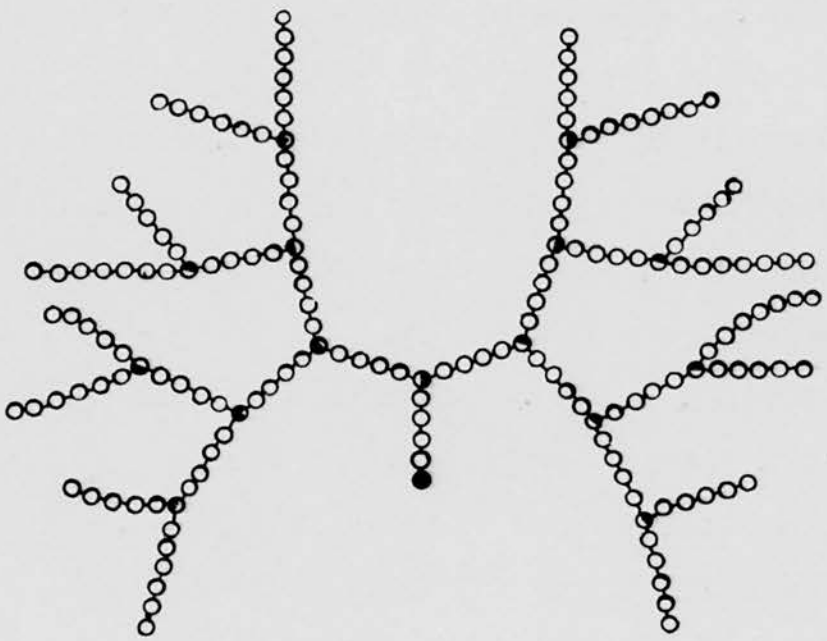
through the same type of bond. The degree of branching varies from 6 - 12%. Here, there is very little doubt that the 1 - 6 branch is practically the only type present, as Bell and Manners (1954) have shown that these constitute at least 99% of the branch points.

Fine Structure of Amylopectin and Glycogen.

Various branched structures have been suggested for amylopectin and glycogen. The earliest of these are the laminated structure of Haworth, Hirst and Isherwood (1937) and the "herring-bone" structure of Staudinger and Husemann (1937). Later, Meyer and Bernfeld (1940), proposed a ramified "tree-like" structure (Fig. |). Enzymic degradation studies have provided the most conclusive evidence to date (Larner et al 1952). This work depends on the successive use of two enzymes; (1) Phosphorylase - which removes glucose from the external chain (as glucose - 1 - phosphate) to within a few units of a branch-point - and (2) Amylo - 1-6- glucosidase - which will remove exposed 1 - 6 linked glucose units. Thus, by the action of phosphorylase on either amylopectin or glycogen, a limit dextrin is obtained which, by treatment with amylo - 1-6- glucosidase, is rendered susceptible to further attack by phosphorylase. By this method, Larner et al were able to obtain from glycogen, a series of 4 limit dextrins of successive degrees of conversion 36%, 62%, 77% and 88%.(Fig.). Similar results were obtained for amylopectin. These could only be interpreted in terms of a "tree-like" structure. From these results an idealised "tree" structure was defined as one in which the number of units between branch-points is constant and

FIG. 1.

"Tree" structure for glycogen



● Reducing end group

⊙ Branch point

in which the number of branch-points doubles in every tier. On this basis the molecular weight can be calculated as

$$M = (2^n - 1) \times m$$

where n is the number of tiers and m is the molecular weight of the chain between.branch points.

Madsen and Cori (1958) have suggested that as a consequence of this structure, glycogen must have a limiting molecular size. It is easily shown that, because of the rapid increase in the number of branches, the surface area available to the end of each chain decreases steadily with increasing molecular size. A simple calculation showed that, for a molecular weight of 20 million, the available area was barely sufficient to accommodate the chains at the surface. These authors therefore suggested that this must be the maximum possible size of such a structure. However, it seems rather unreasonable to apply a purely hypothetical model so rigorously; a proportion of the chains could obviously terminate within the molecule. In this way, a considerably increased size would be possible. In any case, the area available to the growing chains would probably not be the size-limiting factor, since these molecules are synthesised by enzymes which must gain access to the growing chain. The enzyme concerned, phosphorylase, is of a much larger size than the chain (M.W. ~ 500,000) and this factor is, then, more likely to set a limit upon the size. Thus, although in general outline this structure is correct, it is probable that neither amylopectin nor glycogen conforms completely.

Molecular Weight Distribution in Amylopectin and Glycogen.

Weight average molecular weights ($\overline{M}_W$) for both branched glucosans are high (several millions) but number average molecular weights ($\overline{M}_N$) are usually lower by quite a large factor. This wide distribution of molecular weights is confirmed by the width of the sedimentation patterns found on ultracentrifugation.

Erlander and French (1956) have attempted to explain this extremely large spread of molecular weight by a statistical theory of the formation of such branched polymers. The process of formation is considered as a random condensation polymerisation of multi-functional units, where each functional group has a definite individual probability of reaction. In these natural polymers, the glucose unit is tri-functional, the C_4- having a greater probability of reaction than the C_6-position. By defining certain fundamental restrictions, Erlander and French were able to give a statistical formulation of molecular weight distribution. These restrictions were

(1). C_1 can react only with C_4 or C_6.

(2). No ring formation may take place.

(3). There must be one, and only one, unreacted C_1 per molecule.

The resulting molecular weight distribution was extremely wide with the ratio of $\overline{M}_W / \overline{M}_N \to \infty$ as $\overline{M}_N$ increased. Thus, for glycogen with 10% branching, a weight average of 5×10^6 corresponds to a number average of only $1{\cdot}26 \times 10^5$. This weight average is approximately that found in the literature for liver glycogen, but

most experimental number average molecular weights lie in the range $0{\cdot}5 - 2 \times 10^6$. Further, since most $\bar{M}_N$ determinations are made by osmotic measurements, which are notoriously insensitive in this region, these results are probably underestimated. Thus it would appear that, in practice, distributions are rather narrower than predicted by this theory. However, glycogen usually undergoes several precipitations with alcohol during isolation, and this might result in the loss of a proportion of the smallest material. In addition, many workers (e.g. Cori 1956) purify glycogen by rejecting material precipitated by low concentrations of alcohol, thereby losing some high molecular weight material. Both of these procedures, therefore, tend to narrow the distribution.

A further complication in the comparison of experimental results with theory is the strong probability that degradation takes place during isolation. Erlander and French suggest that, if such degradation were purely random, the theoretical distribution would still apply, as the degradation would merely reverse the synthesis. However, Pollard (1957) has suggested that in the "tree" structure, the initial branch is under considerable strain and is therefore most liable to degradation. This strain is presumed to increase with molecular size, the largest molecules being least stable. A narrower distribution would result from such degradation. Thus it seems improbable that this theory will apply rigorously to experimental products, although it does serve to emphasise the wide distribution liable to be found.

However, the fundamental assumption of this hypothesis might be questioned. These authors have assumed a completely random synthesis

by what is effectively a "multi-chain" mechanism. While this may be the case in homogeneous condensation polymerisations as, for instance, the synthesis of polyglucoses by Mora (1957), it seems doubtful whether it can be so for in vivo syntheses. Natural synthesis is accomplished by enzyme systems which are probably localised at specific points in the organism. In the starch granule, in particular, a high degree of order is apparent and growth appears to take place at the surface. Under such conditions, a "single-chain" mechanism, in which each molecule is synthesised completely at one contact with the enzyme, is more probable. This would be energetically advantageous as the activation energy could be retained and thus effectively be lowered. The distribution resulting from this type of synthesis would probably be somewhat narrower since there would be less material of an intermediate molecular weight. In this context, some recent work by Bovey (1959) on the in vitro synthesis of dextran - a similar polysaccharide - is interesting. He showed that the weight average molecular weight increased very little over degrees of monomer conversion ranging from 5 - 100%. These results were in complete accord with the "single-chain" mechanism. However, in this work too, very wide distributions were present. Thus, although it is not possible to predict with any accuracy the molecular weight distribution, this must inevitably be very wide in any highly branched polymer.

The foregoing summary of current theories in the chemistry of the starch-type polysaccharides suggests that many problems still require clarification. Our present state of knowledge is such that further progress may be made only through application of biological or physico-chemical techniques. This thesis is primarily concerned with the latter approach.

SECTION II.

PHYSICAL TECHNIQUES.

II A. LIGHT SCATTERING.

The phenomenon of light scattering has been known for many years and its theoretical foundations are well-established. However, only in the last two decades has application to the study of colloidal solutions made it of very much wider practical significance. Light scattering was first used in this way, to determine the molecular weights of proteins, by Putzeys and Brosteaux in 1935. This work was purely empirical in approach and extended practical applications had to wait the developement of the necessary theory. Starting with the work of Debye (1944), many other workers (see review by Doty and Edsall - 1951) have developed the theory required to deal with most cases of interest. Concurrently, the design of equipment has also reached a high level, although some aspects have not received quite enough attention and problems - notably in the field of preparation of solutions for measurement - still remain.

THEORY.

When light traverses a medium, several phenomena may occur.

(1). Part of the light may be absorbed, its energy re-appearing as fluorescence, Raman Spectra or heat.

(2). The light may be scattered.

For the former to occur, the incident radiation must correspond to a natural frequency of the molecules of the medium and this effect is thus dependent upon their chemical nature. Light scattering, on the other hand, is essentially an optical diffraction phenomenon in which

the particles of the medium act as secondary sources of radiation of the same frequency. It does not, therefore, give any information regarding chemical structure directly, but only inasmuch as this may be reflected in the physical size and shape of the particles.

Small particles.

Rayleigh (1871) first calculated the radiation scattered by a single small isotropic sphere, by considering the oscillating dipoles induced in it by the incident electromagnetic field. The magnitude of the secondary radiation resulting, can be expressed in terms of the incident intensity and the polarisability of the particle.

Thus for unpolarised light $$I_{(r)} = \frac{8\pi^4\alpha^2 I_o (1+ \cos^2 \theta)}{\lambda^4 r^2} \quad (1)$$

where $I_{(r)}$ is scattered intensity at distance "r"

$I_{(o)}$ is incident intensity

α is polarisability

λ is wavelength

θ is angle of observation i.e. angle between I_r and I_o

This equation can be somewhat simplified by defining the Rayleigh Ratio - R_θ as

$$R_{\theta} = \frac{I_r \times r^2}{I_\theta} \quad (2)$$

Hence $$R_\theta = \frac{8\pi^4\alpha^2 (1 + \cos^2 \theta)}{\lambda^4}$$

The term $(1 + \cos^2\theta)$ is introduced because the incident light is unpolarised. Incident light polarised vertically to the plane of observation is scattered symmetrically and R_θ is independent of angle. The scattering of horizontally polarised light is

proportional to $\cos^2 \theta$.

The simplest possible case of practical interest is the scattering from a dilute gas. Since here the particles are completely independent of each other, the total scattered intensity is merely the sum of the individual contributions. A dilute solution of small isotropic spheres can be treated in the same way.

$$\text{Hence} \quad R_\theta = \frac{8\pi^4 \alpha^2}{\lambda^4} v(1 + \cos^2 \theta)$$

$$\text{where} \quad v = \text{no particles / cc.} = \frac{Nc}{M}$$

N = Avogadro's No., c = concentration in gm/cc. and M = molecular weight.

Further it can be shown that

$$\alpha = \frac{n_o M}{2\pi N} \left(\frac{dn}{dc}\right)$$

where n_o is refractive index of solvent.

$$\text{and therefore} \quad R_\theta = \frac{2\pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2 Mc}{N\lambda^4} (1 + \cos^2\theta)$$

$$= K c M (1 + \cos^2\theta) \qquad 3.$$

$$\text{where } K = \frac{2\pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2}{N\lambda^4}$$

As the dilute solution has been treated as a gas, this scattered intensity is that derived from the solute particles alone; that is the excess scattering of solution over solvent.

For more concentrated solutions, or for a pure liquid, the movement of particles can no longer be considered as independent and a certain degree of order exists. Destructive interference therefore occurs among the radiation scattered from different

molecules - intermolecular interference - resulting in a decrease in scattered intensity. If the medium were completely homogeneous, no light at all would be scattered. The scattering from a concentrated solution can therefore be calculated in terms of its departure from complete homogeneity. This is usually known as the "fluctuation method", since it is based upon calculation of the probable fluctuations of concentration from its mean value, by consideration of the thermodynamic free energy changes involved. The result of this calculation is

$$\frac{Kc}{R_\theta}(1 + \cos^2\theta) = \frac{1}{M} + 2A_2c + \text{higher terms in c.} \quad (4).$$

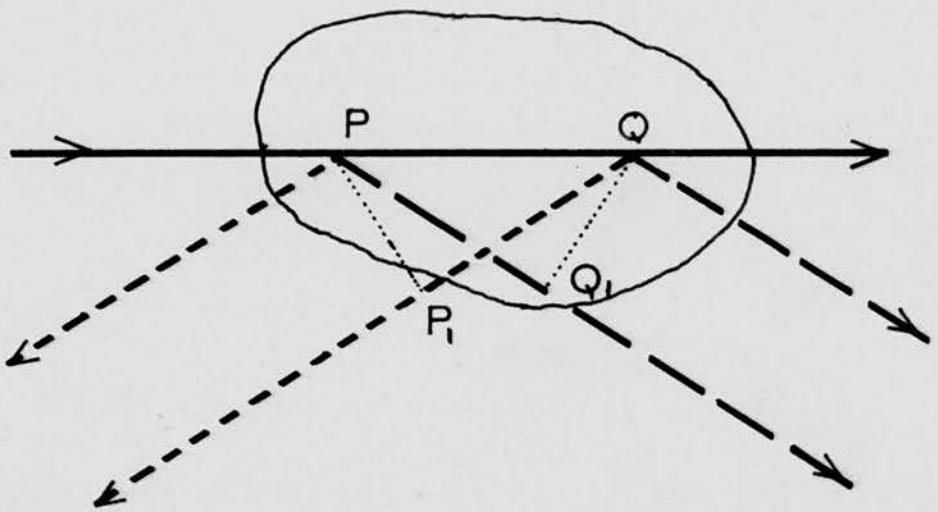
where A_2 - the second virial coefficient - has the same significance as in osmotic pressure theory.

Large particles.

In the above discussion, the particles have been assumed to be small in comparison with the wavelength of the light, so that they may be treated as point sources. This approximation is only valid for dimensions less than $\frac{1}{20}\lambda$. When, however, a dimension of the particles exceeds $\frac{1}{20}\lambda$, light scattered from different parts of the same particle can interfere due to differences in path length. This leads to a reduction in scattered intensity, which is, however, not symmetrical. As can be seen from Fig. 2, a greater phase difference is possible in the backward than in the forward direction. This departure from symmetry will obviously increase with particle size and is also found to be dependent on the actual shape of the particle. Debye (1947), Zimm, Stein and Doty (1945) and others have

FIG. 2

Scattering from a large particle



Path difference

Forward

$PQ - PQ_1$

Backward

$PQ + QP_1$

calculated this effect for various simple models. These are spheres, random coils and rods, Of these, the most important to polymer chemists is the random coiling chain and the theory of this model has been treated very extensively by Zimm (1948), Flory (1958), Hermans (1958) and Hyde et al (1958).

The Particle Scattering Factor, P_θ, is defined as the ratio of the observed intensity to that in the absence of interference. P_θ for the simple models is shown below.

$$\begin{aligned} &\text{Spheres} \quad P_\theta = \left[(3/x^3) \times (\sin x - \cos x)\right]^2 \\ &\text{Coils} \quad P_\theta = (2/y^2) \times \left[\exp(-y) - (1-y)\right] \qquad (5). \\ &\text{Rods} \quad P_\theta = (1/u) \times \int_0^{2u} (\sin w/w)\, dw - (\sin u/u)^2 \end{aligned}$$

Where

$$x = \frac{kSD}{2} \quad ; \quad y = \frac{k^2 S^2 R^2}{6} \quad ; \quad u = \frac{kSL}{2}$$

D = Diameter of sphere; R = root mean square end-to-end distance of coil; L = Length of rod.

$$k = 2\pi/\lambda' \quad ; \quad S = 2 \sin \frac{\theta}{2}$$

λ' is the actual wavelength of light in the solution and is given by

$$n\lambda' = \lambda_o$$

Equations (5) may be employed in two ways to determine the molecular weights of large molecules.

(1) The dissymmetry method (2) The extrapolation method.

(1). The dissymmetry method. This is based upon the fact that P_θ, for all models, decreases smoothly from unity at 0°. Thus the ratio of the intensities at two angles will completely define the scattering and hence the dimension for a particular model. In addition, since

$$P_\theta = \frac{R_\theta(\text{obs.})}{R_\theta(\text{ideal})}$$

$$\frac{K c}{R_\theta} = \frac{1}{M P_\theta} \qquad (6).$$

A light scattering determination usually involves the measurement of R_{90°. If the scattering is not symmetrical, the ratio of intensities at two other angles is also required. The angles most often selected are 45° and 135°. The ratio $I(45^\circ) / I(135^\circ) = Z$ is known as the "dissymettry ratio". From the value of Z, P_{90° is obtained using equations (5) or tables derived from (5) by Doty and Steiner (1950). This, then, allows the determination both of molecular weight and size but requires either a knowledge or an assumption of the molecular shape. This method is very simple, but quickly becomes inaccurate as the ratio $\frac{D}{\lambda}$ increases, since the values of P_θ for the various models differ considerably. For larger values of $\frac{D}{\lambda}$, it is more accurate to use the second method.

(2). The extrapolation method. P_{0° for all models is unity, and therefore if the value of R_{0° can be established, the molecular weight can be unambiguously determined. It is obviously not possible to do this directly, but from measurements made at sufficiently low angles, a reliable extrapolation to 0° can be made. Following Zimm (1948), this extrapolation can best be made

by plotting Kc/R_θ as a function of $\sin^2 \frac{\theta}{2} + k'c$; where k' is an arbitrary constant chosen to spread out the data suitably. A grid-like graph is obtained (Fig.17) and extrapolation made along lines of constant angle and constant concentration simultaneously to a common intercept upon the Kc/R_θ axis. This intercept, $(\frac{Kc}{R_\theta})_{\substack{c=0 \\ \theta=0}}$, is equal to the reciprocal of the molecular weight (equation 6). Further useful information can be obtained from the slope of the zero concentration line, since it can be shown that

$$\frac{\text{Initial slope of zero conc. line}}{\text{Intercept}} = \frac{d(\frac{1}{P_\theta})}{\sin^2 \frac{\theta}{2}} = \frac{16}{3}\pi^2\left(\frac{\rho_g}{\lambda}\right)^2 \qquad (7).$$

where ρ_g - the radius of gyration - has a meaning similar to its more usual one in mechanics and is given by

$$\rho_g^2 = \begin{cases} \frac{3D^2}{20} & \text{for spheres} \\ \frac{L^2}{12} & \text{for rods} \\ \frac{R^2}{6} & \text{for coils} \end{cases} \qquad (8).$$

The slope of the zero angle line defines the second virial coefficient A_2. It has been shown by Holtzer, Benoit and Doty (1954), that over a limited range of size, the zero concentration line is curved. The intercept of the lower portion defines the weight average molecular weight $\bar{M}_w$ as in the normal case. However the intercept of the asymptote to the upper part of the

curve is equal to 1/2 $\bar{M}_n$ and therefore gives a number average molecular weight. The size range over which this effect occurs is very limited and hence it is seldom of any practical significance.

This extrapolation method, then, allows the determination of molecular weight and size for molecules with dimensions approaching the wavelength of the light used. For even larger molecules, this simple theory breaks down, and in order to explain the observed scattering of such molecules, it is necessary to use the extremely complex general theory of Mie (1908). However, this theory is seldom necessary on dealing with solutions of polymers, either natural or synthetic.

Loss of intensity of primary beam.

The foregoing treatment is based on the angular distribution of the scattered light. An alternative approach is to consider the reduction of intensity of the transmitted beam. A conventional absorption coefficient may be defined

$$\tau = \frac{1}{L} \cdot \log_e \frac{I_0}{I} \qquad (9).$$

τ is known as the turbidity of the solution. In the ideal case, the turbidity is related to the Rayleigh Ratio by

$$\tau = \frac{16}{3} \pi R_{90^\circ} \qquad (10).$$

Hence equation (3) may be written $\tau = \frac{16}{3} K c M$

$$= H c M \qquad (11).$$

For the ideal case $\tau \propto \frac{1}{\lambda^4}$

When, however, the scattering becomes unsymmetrical, this

no longer holds. Doty and Steiner (1950) have shown that

$$-\frac{d(\log \tau)}{d(\log \lambda)} = 4 - \beta \qquad (12).$$

where β can be ezpressed in terms of the dimensions of the solute particles.

This method is seldom used to determine molecular weights but is extremely useful for calibration purposes.

Depolarisation. The usual relationship between angle of observation and state of polarisation of the scattered light has been given above. In particular, there should be no horizontally polarised component in the light scattered at 90°. However, for anisotropic materials this no longer holds. This effect may be corrected for [Cabannes and Roccard (1929)]. However, it is seldom of great importance for polymeric materials unless these are very anisotropic as, for instance, molecules of rod-like structure. In practice, it is extremely difficult to determine depolarisation unambiguously, as the degree of convergence of the light, presence of dust and secondary scattering may affect the measured values [Gans (1929), Rousset (1936), Doty (1948), Gueidushek (1954)]. In measurements on pure liquids, or very small solutes, (e.g. sucrose), very large corrections - ~20% - may be required.

II. LIGHT SCATTERING IN PRACTICE.

The previous section has indicated the two general methods by which measurement of molecular weight can be made - the measurement of (1) Turbidity or (2) The Rayleigh Ratio. Both are generally applicable; the first having the advantage that standard spectrophotometers may be used, whilst the second requires much more specialised equipment.

Turbidity.

As the measurement of turbidity involves the comparison of two very nearly equal intensities, its application is restricted to high concentrations of large molecular weight material. On the other hand, the measurements are absolute and hence are of considerable value in calibration of the second method, which is more generally used.

The Rayleigh Ratio.

This method, while founded more soundly in theory, is much more difficult experimentally as it involves the measurement of very low intensities and their comparison with the much greater intensity of the incident beam. For the scattered light, detectors of the highest possible sensitivity are required, but the incident intensity may be greater by a factor of 10^4. All practical methods necessarily use photomultiplier tubes for measurement of intensity of scattered light, but differ in their accommodation of the greater intensity of the incident light. Several inatruments [Zimm (1948), Peaker (1952)] employ a

second, less sensitive, photocell, whilst others [Debye (1944), Brice, et al (1950)], use some form of attenuation in the incident beam. The observed ratio of two intensities must then be related to the Rayleigh Ratio. For this correlation to be absolute, careful consideration of the geometry of the instrument is required. In particular, in the 90° position, the phototube views an "extended source", whilst it receives essentially parallel light in the 0° position. In the Brice instrument [Brice, et al (1950)], this difficulty is avoided by introducing an opal diffuser into the incident beam while measuring its intensity, thus making the geometry of viewing effectively the same for both measurements. A further complication occurs through refraction effects at cell windows which reduces the actual cone of light "seen" by the phototube. Various authors [Carr and Zimm (1950), Brice et al (1950) (1954), Hermans and Levinson (1951)], have examined this effect, and have shown that, in general, it is dependent upon cell shape and upon the refractive index of the scattering medium. However, for the particular case where the viewing system is constructed so that the phototube cannot "see" past the edges of incident beam in the vertical direction, Hermans and Levinson (1951), have shown that the necessary correction factor is given by n^2 for all cell shapes. This has been confirmed experimentally by Meier (1953). By careful analysis, Brice et al were able to evaluate all the factors involved in this absolute calibration of their photometer, but in practice it is probably much more satisfactory to calibrate by a relative method. The latter

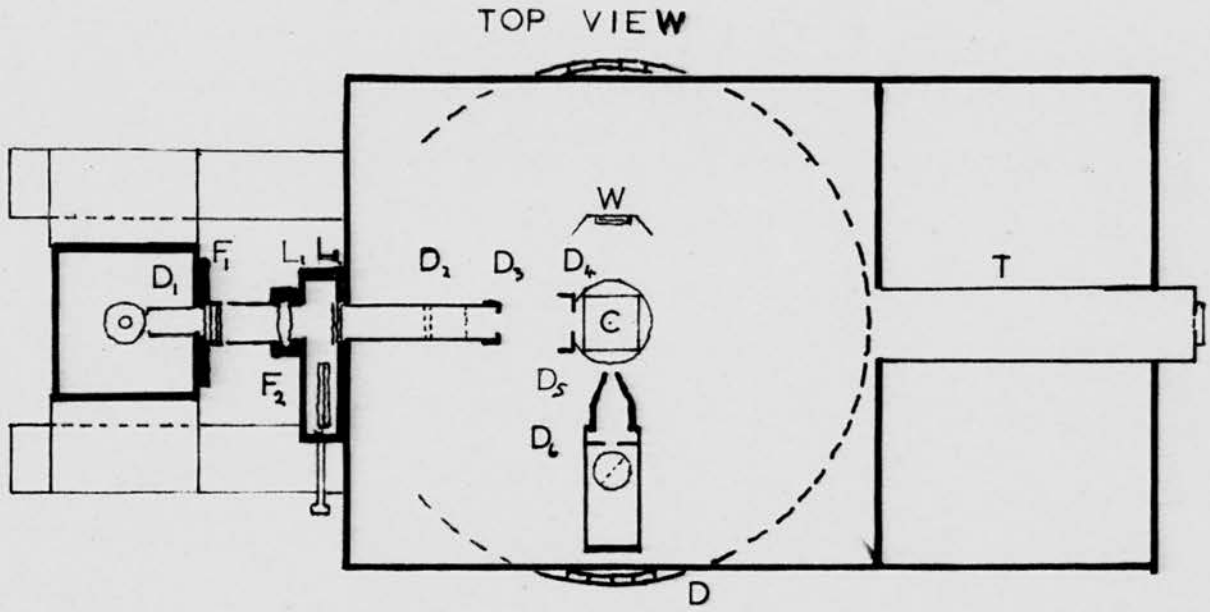
involves the comparison of the experimental ratio with the Rayleigh Ratio deduced from absolute measurements of turbidity with a spectrophotometer.

The photometer designed by Brice et al, is thus of good general utility and as it is produced commercially by the Pheenix Precision Instrument Co. , Philadelphia, U.S.A., it has been used in this work. A brief description is given below.

Description of the Photometer.

The optical system is shown diagrammatically in Fig. 3 Monochromatic light is selected from a mercury vapour lamp by the filter F1 and is collimated by the lens system L_1, L_2. The intensity can be reduced by the 4 neutral filters, F2. The diaphragms D2, D3 and D4 produce a parallel beam of well-defined dimensions. The cell containing the experimental solution is fitted on the cell table C, recessed to accept different types of cell. The photomultiplier housing is set upon an arm, rotatable about the centre of the cell table by means of a graduated disc D. The viewing angle of the phototube is defined by diaphragms D 5, D 6, mounted in a removable nosepiece. On the opposite end of the photomultiplier arm, is mounted an opal glass diffuser - the working standard W, which is in position in the incident beam in the 0° position. This serves partly to reduce the intensity, and partly to produce viewing conditions similar to those at 90°. The incident beam, after passage through the cell, is trapped in the blackened tube, T. The high voltage D.C. supply to the photomultiplier from a stabilised

FIG. 3



LIGHT SCATTERING

PHOTOMETER

power source, is via a potentiometer network which allows the adjustment of sensitivity. The output from the photomultiplier is measured, without amplification, by a galvanometer of sensitivity 1,400 mm/μA. The instrument is very stable, the short-term stability being rather better than 1%.

Measurement of Scattering Ratio - With the cell in position, the photomultiplier is set at the required angle and the sensitivity adjusted to give a high deflection with all neutral filters withdrawn. With all filters inserted, the arm is turned to 0° and sufficient filters withdrawn to give a suitable reading. An alternative, and more convenient, method is to adjust the sensitivity to the 0° position with all filters inserted and then to remove them as required in the angular position. In either case, the observed reading (g) is divided by the combined transmittance (see p. 36) of the neutral filters employed.

$$G = g/F$$

The ratio $\frac{G_\theta}{G_{0^\circ}}$ is the experimental scattering ratio which must be related to the Rayleigh Ratio, either by a geometrical calibration or by a relative method (see p. 30).

The calibration factor, based upon the analysis of Brice, quoted by the makers is

$$R_{90^\circ} = k\,(n^2\,\frac{R_w}{R_c})\,a\,\frac{G_{90^\circ}}{G_{0^\circ}}$$

where k = 0·0632 at 436 mμ

= 0·068 at 546 mμ

n = refractive index of solution.

"a" is a constant relating the transmittance of the working standard to that of a standard opal diffuser - determined experimentally at frequent intervals.

$\frac{R_W}{R_C}$ is a factor correcting for residual refraction effects, dependent upon refractive index.

Values for various solvents are given in Table.

Solvent	n	$n^2 \frac{R_W}{R_C}$
Methanol	1·332	1·88
Water	1·337	1·91
Butanone	1·383	2·05
Benzene	1·511	2·52

The determination of filter transmittances and working standard constant is detailed in experimental section.

Reflection Effects in Angular Measurements.

Angular measurements of light scattering are complicated by the occurrence of reflection effects at interfaces. These are considered here as a preliminary to a discussion of cell design. When light is incident upon an interface between two media of differing refractive index, a proportion is always reflected - Fresnel reflection. As this occurs at all the interfaces in a scattering cell, the effect must be considered, although for symmetrical scattering it is of no importance.

The proportion reflected at an interface between media of refractive index n, n_o is given by

$$R = \left(\frac{n - n_o}{n + n_o} \right)^2$$

Fig. 4 shows the position at the exit window. Here "r" is the fraction reflected at the liquid/glass interface and R' that at the glass/air interface. For unit incident intensity, it should be clear that the total "back reflected" beam R_T is given by

$$R_T = (1-r)^2 R' + r$$

The intensity observed is then $I'_\theta = I_\theta + R_T I_{(180-\theta)}$

and the dissymmetry

$$Z = \frac{I_\theta}{I_{(180-\theta)}}$$

$$= \frac{I'_\theta - R_T I_{(180-\theta)}}{I'_{(180-\theta)} - R_T I_\theta}$$

$$= \frac{Z' - R_T}{1 - R_T Z'}$$ (Oth et al 1953).

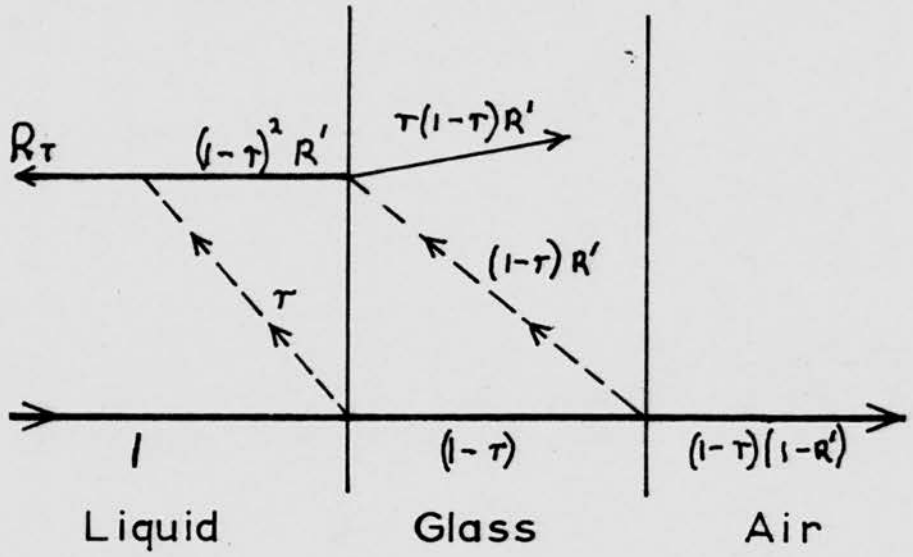
Onyon (1957) has shown that the above correction is not sufficient in the case where reflection may occur at the back surface of the cell. He was particularly concerned with reflection of the 90° radiation in square cells but the same reasoning must be applicable to cylindrical cells. Here a fraction R_T of the light scattered at the angle $(-\theta)$ is reflected to augment the observed intensity at $(180 - \theta)$. The intensity actually observed is then

$$I'_\theta = I_\theta + 2R_T I_{(180-\theta)}$$ (see Fig. 4).

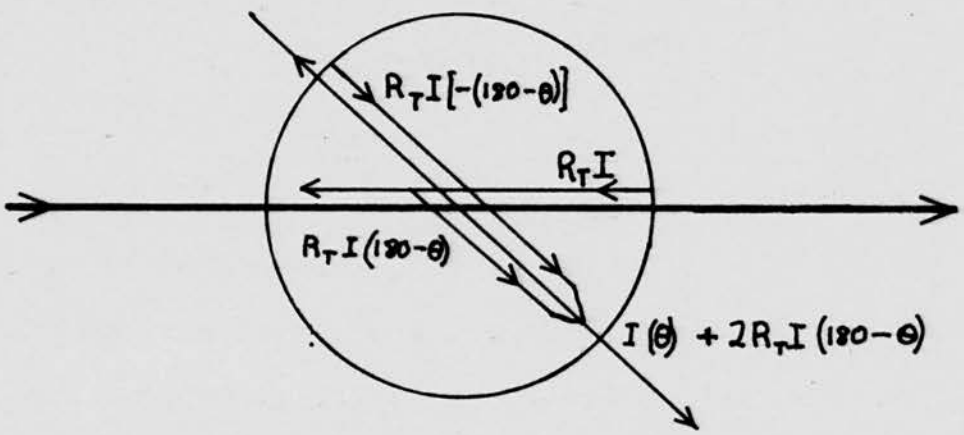
However, in the commercially available cylindrical cells (Phoenix Precision Instrument Co.) the back surface is usually ground, and hence only diffuse reflection will occur. Thus the actual contribution from this effect will be unknown.

FIG. 4

Schematic representation of Fresnel reflection at a cell wall.



Back reflection in a cylindrical cell.



CELL DESIGN.

Square, rectangular, cylindrical and conical shaped cells have been used in photometers but each have inherent disadvantages. The choice of cell is governed by the type of measurements required. These problems are discussed below and the design of a new type of cell is suggested.

The simplest possible shape is square and this can be used satisfactorily where only $R_{90}°$ is required. Johnson and Goring (1952) have used long rectangular cells for angular dependence measurements, although here a correction for further refraction effects is necessary.

When measurements of $R_{90}°$ and $I_{45}°/I_{135}°$ only are required, semi-octagonal cells have often been employed. This type of cell has been found to give rise to strong total reflection from the back corners [Oth et al (1953)]. Further, the placing of these cells is very critical [Mommaerts (1952)], and the value of Z is open to question since $Z<1$ may sometimes be observed.

Measurement of three commercial semi-octagonal cells available to the author, revealed that in no case were the entrance and exit faces parallel, the minimum deviation being $1\frac{1}{2}°$. This can cause a deviation from the primary direction either within the cell or on leaving the cell. In the first case, the deviation of the beam within the cell could be 1° and therefore the observed dissymmetry, instead of being $I_{45}°/I_{135}°$, would be either $I_{44}°/I_{134}°$ or $I_{46}°/I_{136}°$, which, for completely symmetrical scattering, would be 1·036 or 0·965. In both cases, the position

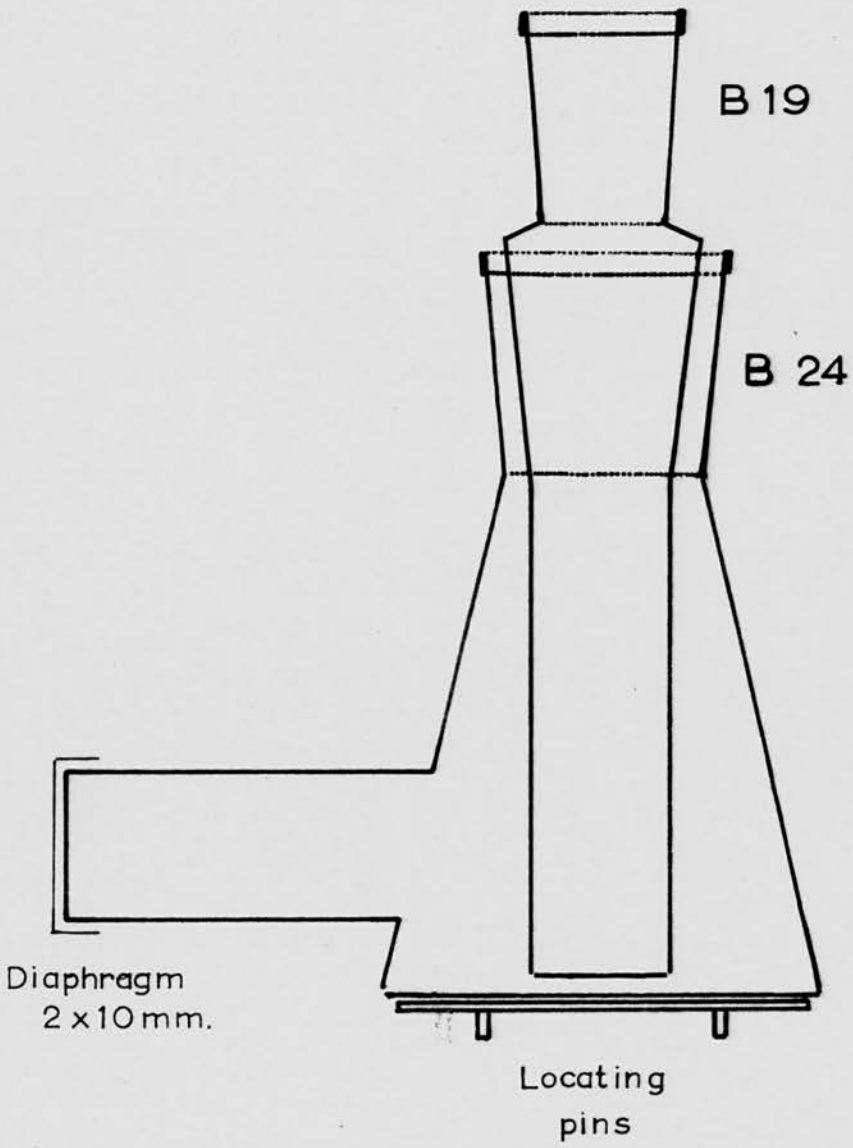
of maximum intensity in the transmitted beam would not be at 0° and hence the measurement of the incident intensity would be in error. This is the more serious fault as, with narrow incident beams, the intensity falls off very rapidly away from 0°, being effectively zero at $\theta > \pm 3°$. For these reasons semi-octagonal cells have not been considered satisfactory in this work. Cylindrical cells, which allow angular dependence measurements to be made without corrections for refraction, are only usable in air if they have flat entrance and exit windows. This type of cell is much more satisfactory as regards spurious reflections although, as indicated above, the value of the correction for reflection at the back surface is doubtful. This, however, may safely be neglected as it can only be small. Although these cells may be prone to the same difficulty of non-parallelism between exit and entrance windows, it has beenfound in this work that when aligned to give the least deviation of the transmitted beam, the error involved is negligible. Many workers have recommended the use of conical cells in order to avoid corrections for back reflection. Light is then reflected downwards and hence out of the volume viewed by the phototube. However, since such cells give refraction in the vertical plane, they cannot be used in air except, as by Zimm (1948), by incorporating a complex optical system whereby the incident light is directed upward at such an angle that it traverses the conical cell horizontally and is then received by the phototube, also placed at an angle. While this does eliminate - almost completely - reflection corrections,

considerable complexity in design is introduced. An alternative is the use of a conical cell immersed in a cylindrical bath [Dandliker and Kraut (1956)]. For this to have any advantage over a simple cylindrical cell, it is necessary for the transmitted beam to be absorbed in a "Wood's Horn" which then does not allow the measurement of incident intensity without removal of the entire assembly. Also, the reflections at the back surface are not completely eliminated, although this surface can be blackened. Thus for the complete elimination of reflections the conical bath (Zimm) is essential, but the complex optical arrangement makes it difficult to apply except where the photometer can be designed around it. The use of a conical inner cell has no particular advantage as the reflections at glass/liquid interfaces are small.

The optical complexity associated with the conical cell can be avoided if the entrance window is made perpendicular to the incident beam. Such a cell was constructed from an 100 ml. conical flask, by attaching a side arm carrying a flat vertical window (Fig. 5). The solution was contained in an inner cylindrical cell. This was supported centrally on a B 24 ground joint. A slight disadvantage accompanies this design in that the light leaving the cell is deflected downward. However, the deviation is only slight and results for any one solvent are consistent, (see p. 39).

FIG. 5

CONICAL LIGHT SCATTERING CELL



For any design of cell, it is necessary to be able to show that no distortion of the light path is produced. Further, as the volume "seen" by the phototube varies with angle, this variation must also be determined. Simple geometrical considerations show that the volume "seen" should vary inversely with sin θ, and hence, any departure from this relation indicates distortion and/or bad alignment. As the attainment of a perfect system is extremely difficult, it is preferable to determine a correction. This can be simply obtained by measuring the fluorescence of a dilute solution of fluorescein. The intensity envelope for this fluorescence is symmetrical and hence the observed intensity should be inversely proportional to sin θ. To avoid interference from scattered light, the solution is illuminated with blue light and the phototube screened with a yellow filter passing only the green fluorescence, (Brice et al 1950). TableI p.37 shows the results of such an experiment for a cylindrical cell and the cell described above.

CALIBRATION OF PHOTOMETER.

As previously indicated, calibration of the photometer in terms of a standard of known turbidity, is the most satisfactory means of obtaining absolute values of the Rayleigh Ratio. This, in effect, was the method used originally by Putzeys and Brosteaux (1935), who obtained the molecular weights of a number of proteins in terms of another protein, amandin. The weakness of this, and similar methods, lies in the necessity to predict the light scattering behaviour of the standard material from its molecular

weight derived by other methods. Now, as the scattered light is proportional to the weight average molecular weight and most other methods (osmotic pressure, end group, etc.) yield number average molecular weight, an error is introduced. In addition, the possibility of the occurrence of contamination and non-ideal behaviour is not considered. It is therefore, preferable to determine the actual turbidity of the calibrating solution by spectrophotometry and to obtain from this the theoretical Rayleigh Ratio, using the equation

$$R_{90^\circ} = \frac{3}{16\pi}\tau$$

For this method to be applicable, the calibrating medium must fulfil certain conditions.

(1). The particle dimensions must be small compared to the wavelength of light - and hence the dissymmetry must be negligible and $\tau\lambda^4$ must be constant.

(2). There must be negligible depolarisation of scattered light.

(3). There must be no selective absorption.

(1) - (3) are all essential requirements. In use, others may be desirable.

(4). The material should have a high scattering power, i.e. H x M should be large.

(5). Solutions should be easily clarified and reproducible.

The use of a variety of substances has been described in the literature. These include serum albumin and turnip yellow virus,

[Johnson and Goring (1952)], polymethacrylic acid [Alexander and Stacey (1955)] and Ludox" [Mommaerts (1952), Maron and Lou (1954)]. "Ludox" has been widely employed. This material is a colloidal suspension of silica (produced by Dupont de Nemours) which consists of spherical particles of diameter 400 Å [Oster (1952)] and therefore with no dissymmetry. The depolarisation is negligible and there is no selective absorption. The scattering power is high [H x M = 0·02 at 5460 Å - Maron and Lou (1954)]. However, condition (5) is not satisfied. It was found in initial experiments that clarification could not be achieved by filtration as the silica particles rapidly, and permanently, block sintered glass filters. Further, solutions of "Ludox" sometimes show a slow aggregation and the turbidity has to be re-determined in every instance. For these reasons, "Ludox" was discarded as a calibration material.

In the course of the work on glycogen described in Section III, it was observed that many small glycogens had low dissymmetry. If from such a glycogen the higher molecular weight fractions were removed, it might well prove ideal as a calibrating medium. The conditions (1), (2), (3) and (5) would be fulfilled and the scattering power would probably be at least as good as that of "Ludox".

Further, glycogen has the considerable advantage over "Ludox" that it may be stored as a dry solid and solutions of known concentration prepared accurately to give a reproducible turbidity. Thus it is only necessary to determine the turbidity by transmission once over the usable concentration range. This is very convenient

as the determination of the spectrophotometric turbidity requires even greater care over the preparation of solutions to exclude dust than do R_{90}' measurements. Although some error may be introduced by preparing a fresh solution for each calibration without actually determining its turbidity, this is probably no greater than that incurred in transferring solutions from spectrophotometer cell to light scattering cell. The preparation and use of a glycogen for calibration is described in the experimental section.

CLARIFICATION.

Probably the most serious practiaal difficulty in light scattering is the clarification of solutions prior to measurement. In most cases, the scattered light is of very low intensity and therefore extraneous scattering must be reduced to a minimum. This entails the removal of all dust particles from the solution and also, in some cases, of gel material. The contribution to the scattering from these sources is particularly disturbing as these particles are of large dimensions and hence produce very unsymmetrical scattering.

Clarification has been tackled in a variety of ways, all of which may be successful in specific cases. No universally applicable method has yet been devised although all methods are based on either filtration or centrifugation. Most workers have found that, in general, solutions in organic solvents are rather more easily clarified than aqueous solutions. This applies equally to the pure solvents. Organic solvents can be readily clarified by several stages of distillation, but this is not usually effective with water, which must be filtered through the finest grades of sintered glass or through collodion filters. In this work, it was not found possible to obtain water with no dissymmetry, but a fairly reproducible value of about 1·2 was found for water filtered through Grade 5 sintered Pyrex under gravity. As the scattering from the pure solvent also contains the random reflections from various parts of the apparatus, the dissymmetry has no real significance and can be eliminated by subtracting the solvent scattering ratios from those of the solutions.

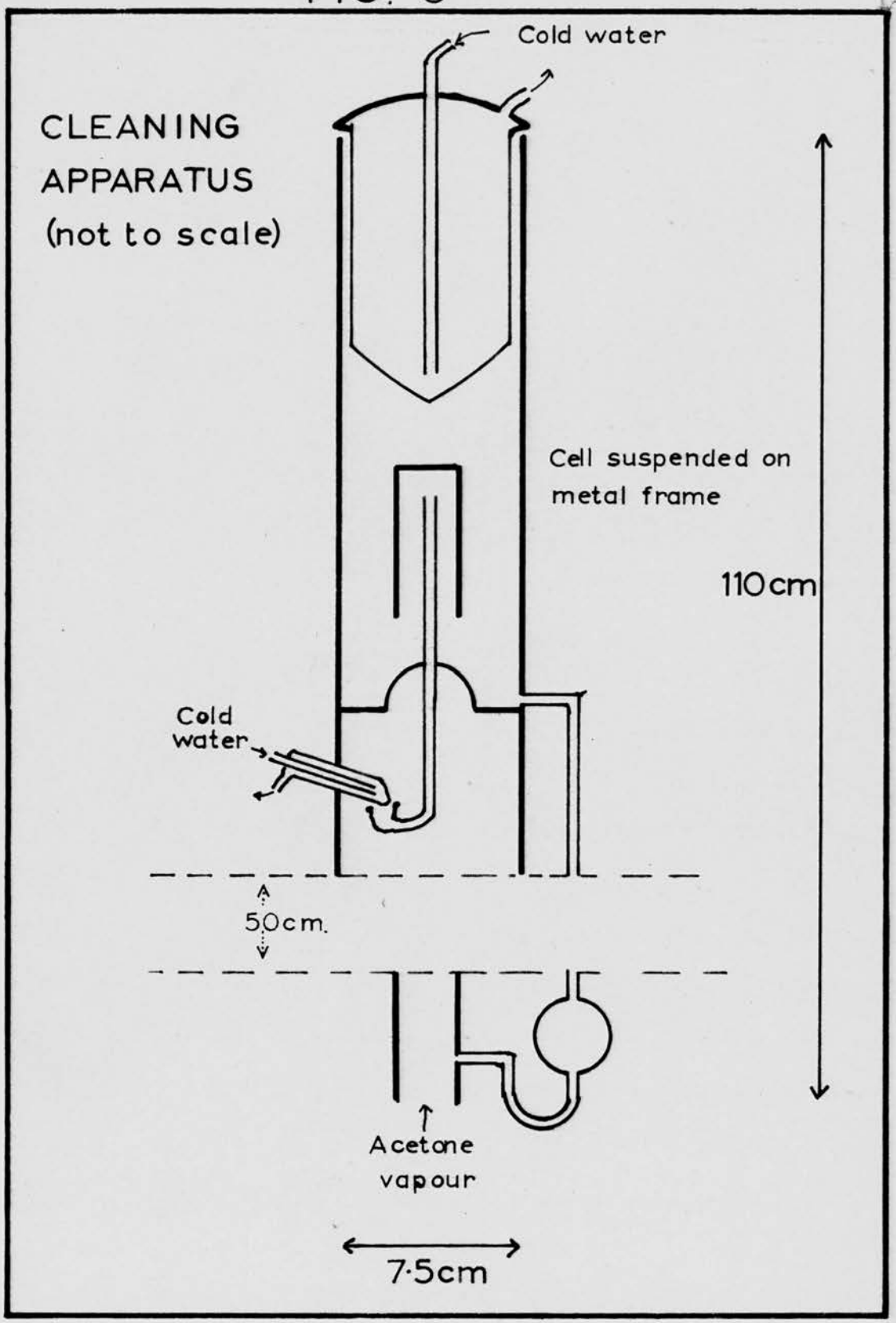
In polymer solutions, it is frequently the case that the solute particles are of comparable dimensions to the dust particles. Thus any method of filtration or centrifugation must also tend to remove solute. It is therefore necessary, perhaps, to suffer a lesser degree of clarification to avoid loss of solute. However, in such solutions the scattering power is usually high and clarification may not be too critical.

The usual proceedure, then, is to obtain the cleanest possible solvent and to clarify the polymer solution, either by filtration or centrifugation (or both if necessary) to the highest degree compatible with minimum loss of solute. To produce a dilution series, small additions of polymer solution are made to the clean solvent rather than the reverse. This tends to keep the effect of contamination to a minimum.

The actual means employed to clarify specific materials will be discussed in the relevant sections.

A necessary accompaniment to clarification is the elimination of dust from scattering cells and other glassware. This was achieved in the present work by suspending the vessels in a stream of condensing acetone vapour in an apparatus similar to that of Thurmond (1952), shown in Fig. 6 . This was preceded by a thorough wash with detergent and rinse with distilled water.

FIG. 6


CLEANING
APPARATUS
(not to scale)
Cold water
Cell suspended on
metal frame
110cm
Cold
water
5.0cm.
Acetone
vapour
7·5cm

EXPERIMENTAL.

(1) Determination of Transmittance of Neutral Filters.

The photomultiplier was set at zero with the working standard in place. Filters (2), (3) and (4) were inserted and the sensitivity adjusted to give a high reading. Filter (1) was then inserted and its transmittance determined from the ratio of the two readings. The mean of ten pairs of readings was taken.

The ratio of the transmittances of filters (1) and (2) was determined from the ratio of the readings with filters (1), (3) and (4) and (2), (3) and (4) in place. Hence the transmittance of (2) could be obtained.

Similarly the ratios (2) / (3) and (3) / (4) were measured and the individual transmittances determined, as shown.

f_1	f_2	f_3	f_4
0·4865	0·2870	0·1330	0·05322

For any experimental combination of filters, the effective transmittance is merely the product of the separate transmittances of the filters.

(2) Determination of the Working Standard Constant - (a).

The constant "a" is the ratio of the effective transmittance of the working standard (WS) to that of a reference opal diffusor (RS) placed centrally on the cell-table.

The sensitivity was adjusted to give nearly full scale deflection with no filters and with the working standard in place at the end of the photometer arm set at 0°. The working standard was then removed and the reference standard carefully inserted,

filter (4) had to be used to give a measurable deflection. Then $a = f_4 \, G(WS) / G(RS)$, where G(WS) and G(RS) are the respective readings.

The value of "a" varied with ageing of the components of the instrument and had to be re-determined at frequent intervals. However, over a three-year period, the value remained between 0·070 and 0·076 for $\lambda = 546$ mμ

(3) Test for angular uniformity.

TABLE I Product of G sin θ for fluorescein solution.

θ°	Cylindrical cell.	Conical cell
30	60·0	49·5
40	62·0	49·3
50	61·5	49·5
60	61·7	49·7
70	61·4	49·7
80	61·5	50·0
90	61·0	50·0
100	61·3	49·8
110	61·0	49·4
120	61·0	49·5
130	61·0	49·0

These figures indicate that over the range 30° - 130° both cells behave ideally and therefore angular measurements may be made without the necessity for further correction.

(4) Calibration.

(a) Extraction of glycogen. Glycogen was prepared from Brewers' Yeast by the method of Northcote and Horne (1952). Purification involved three re-precipitations with ethanol and three with glacial acetic acid. Final traces of contaminating protein were removed by shaking a 2% aqueous solution with toluene overnight (Greenwood and Robertson, 1954), and rejecting the emulsified material which collected at the interface. In order to remove large molecular weight components and gel-forming material, an approximately 5% solution was centrifuged at 20,000 g for 90 minutes. This treatment removed a small quantity of brownish material. The pure glycogen was precipitated with ethanol and dried with ethanol and ether.

(b) Determination of Turbidity. Preliminary experiments showed that the optical density of a 1% solution of Brewers' Yeast glycogen (BYG) was about 0·03 at 500 mμ. Therefore, in order to achieve a reasonable degree of accuracy, it was necessary to employ 10 cm. cells in the spectrophotometer. Measurements were made in the Unicam SP500 spectrophotometer at 11 wavelengths between 400 and 600 mμ. To obtain reproducible readings it was found to be necessary to coat the cells with dull black paint, but no systematic difference was observed when apertures of different size (2mm.-9mm.) were placed in the light path. A blank correction was determined with both cells filled with clarified water.

BYG solutions were first filtered through a G4 sintered filter and then through a G5 filter directly into the clean cell. After

measurement, a sample was taken for determination of concentration by hydrolysis and estimation of reducing power (Lampitt et al 1955).

The turbidity (τ) was obtained from the optical density (D) by $\tau = 2{\cdot}303\ D/10$

$\tau\lambda^4$ was found to be constant between 400 and 600 mμ for all solutions measured. The graph of $\tau\lambda^4/c$ against c is shown in Fig. 7.

(c) Determination of Rayleigh Ratio. Solutions of BYG were prepared as above except that dilutions were made directly into the scattering cell from a concentrated clarified solution. The ratio G_{90}/c was plotted against c for each series and extrapolated to zero concentration to eliminate any possible secondary scattering (Kraut and Dandliker, 1955).

The calibration constant (k) was then calculated from

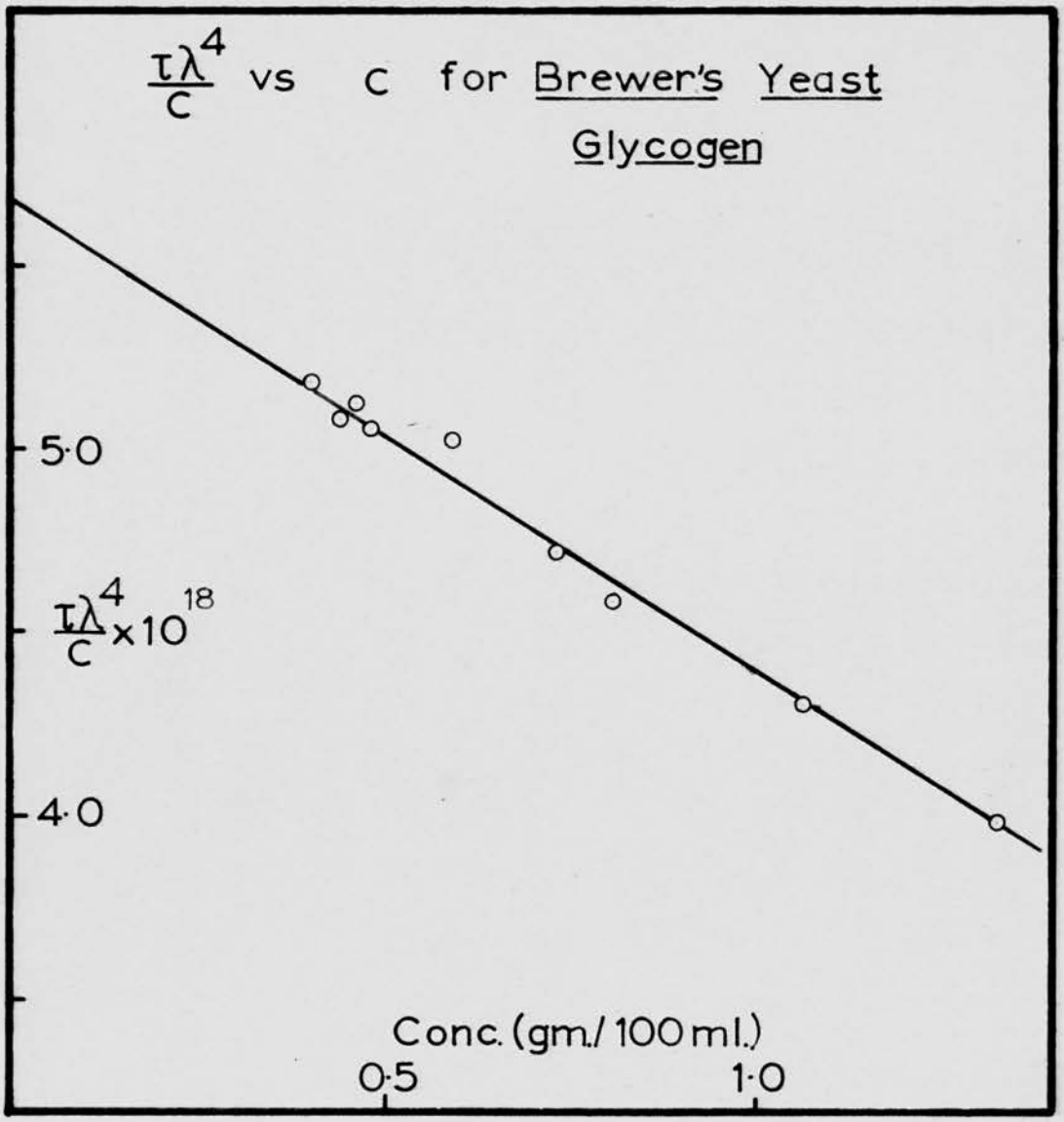
$$k = R_{90}/G_{90}$$
$$= \frac{3}{16\pi\lambda^4}\left(\left[\frac{\tau\lambda^4}{c}\right]_{c=0} \Big/ \left[\frac{G_{90°}}{c}\right]_{c=0}\right)$$

The results of such measurements for a cylindrical cell and the conical cell are given below.

$\lambda = 546$ mμ	Cylindrical cell	Conical cell
	k = 0·0122	k = 0·0182
	0·0124	0·0178
	0·0125	0·0177
	Mean 0·0124	Mean 0·0179

For a square cell, a calibration by this method was compared with the geometrical calibration based upon the opal reference standard.

FIG. 7


$\frac{I\lambda^4}{C}$ vs C for Brewer's Yeast Glycogen
5·0
$\frac{I\lambda^4}{C} \times 10^{18}$
4·0
Conc. (gm./ 100 ml.)
0·5
1·0

Geometrical $k = 0.0090$ Experimental $k = 0.0093$

These results are within experimental error, and hence the geometrical calibration is perfectly satisfactory for the standard square cell, although some form of experimental calibration is necessary when using cylindrical or other non-standard cell shapes.

As a further check on calibration, the 90° scattering of a standard sample of polystyrene distributed by Professor P. Debye was determined in toluene at 546 mμ. The solvent was clarified by distillation through a column filled with "Fenske Helices", and the solutions of polystyrene by filtration through G5 filters. Correction for refractive index was made by multiplying the calibration constant by the ratio $[n(\text{toluene}) / n(\text{water})]^2$

R_{90} for a 0.5% solution was found to be 7.8×10^{-5} compared with the mean of the results of several workers quoted by Carpenter and Krigbaum (1956) as 8.1×10^{-5}.

II B.

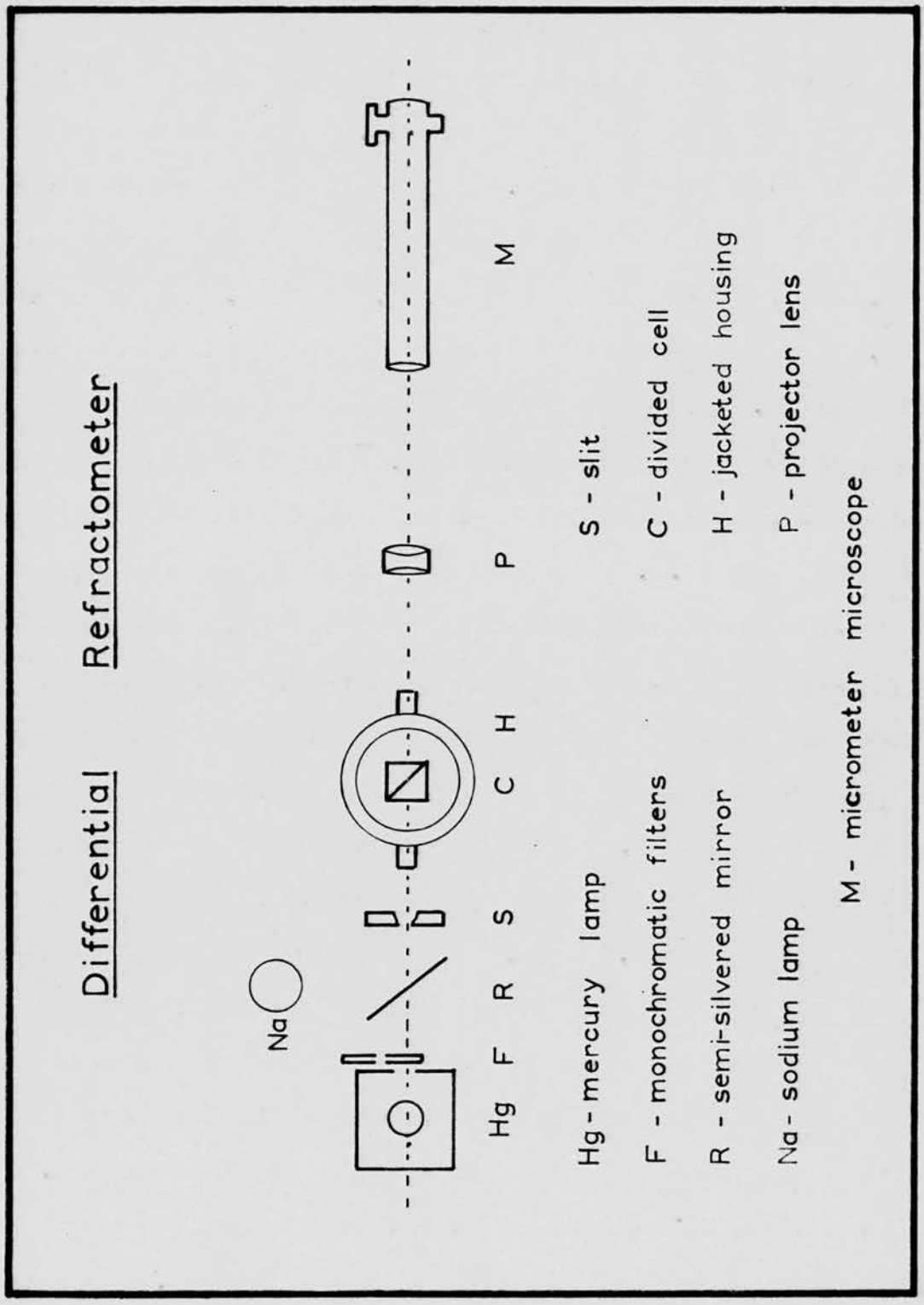
Refractive Index Increment.

For the determination of molecular weight by light scattering, the variation of refractive index of solution with polymer concentration must be known. This factor - dn/dc - appears in the optical constant K as the square. Therefore it must be determined with a high degree of accuracy as any error is effectively doubled. Since, for most systems of interest, the refractive index increment is of the order of 0·150 (ml/gm.) Δn is approximately $1 - 2 \times 10^{-3}$ for a 1% solution. The precision required then is $1 - 2 \times 10^{-6}$. Such accuracy can be attained in some of the high precision absolute refractometers of the Abbé type, but temperature-control must be very good (e.g. dn/dT for water $\sim 10^{-4}$). To avoid the necessity for such good temperature control, an instrument measuring directly the difference of refractive index between solution and solvent is preferable as the temperature dependence of Δn is very small. A Rayleigh interferometer can be used but as this is difficult to operate, many differential refractometers based upon the direct measurement of a deviation have been designed (Brice and Halwer, 1951).

The basic principle of all differential refractometers is that monochromatic light from a slit source passes through a divided cell containing solution and solvent, and the deviation of the beam is measured. The optical arrangement of the refractometer designed by Brice and Halwer and marketed by Phoenix Precision Instrument Co. is shown in Fig. 8

The solution and solvent are each contained in one half of the

FIG. 8


Differential Refractometer
Na
Hg F R S C H P M
Hg - mercury lamp
S - slit
F - monochromatic filters
C - divided cell
R - semi-silvered mirror
H - jacketed housing
Na - sodium lamp
P - projector lens
M - micrometer microscope

divided cell. This is rotatable about its centre through 180° by the arm. By this device the deviation of the image is doubled. The position of the image of the slit is measured with the micrometer eyepiece of the telescope which is graduated in 0 01 mm. Light of wavelength 5460Å or 4360 Å is obtained by filters from the mercury lamp or an external sodium lamp may be used with the half-silvered mirror.

Method.

With solvent in both compartments the displacement of the image between the positions (1) and (2) of the cell is measured. With the cell in position (1), the compartment nearest the lamp is emptied, cleaned and re-filled with the solution to be measured. The displacement is again measured. For all determinations some ten readings of the displacement were made, the telescope being re-focussed for each. In this way the position of the image could be measured to 0·001 mm.

Then $\Delta d = (d_1 - d_2)_{solution} - (d_1 - d_2)_{solvent}$.

Hence $\Delta n = k \Delta d$

where k is a calibration constant determined from measurements on solution of known refractive index.

Calibration.

Sucrose solutions were used for calibration. These were prepared by weight from A.R. grade sucrose which had been dried in a vacuum oven at 70°.

$\Delta n/c$ (589 mμ and 20°) = 0·1436 (Brice et al, 1951)

$\Delta n/c$ (546 mμ and 25°) = 0·1430 (Svensson, 1952).

589 mμ and 20°

Sucrose concentration		k.
0·93%		0·9539
1·50%		0·9539
2·59%		0·9548
4·97%		0·9561
	Mean	0·9547

546 mμ and 25°

3·678%		0·9555
2·45%		0·9550
	Mean	0·9553

II C. VISCOSITY.

One of the most characteristic properties of solutions of high polymers is their high viscosity. Viscosity measurements are simple to make with good accuracy and have, therefore, been very widely used in investigations of polymer solutions.

Definitions.

Absolute viscosities are seldom used in polymer work, the important quantities being relative.

Relative viscosity $-\eta_r-$ Ratio of viscosity of solution (η) to that of solvent (η_0)

$$\eta_r = \eta/\eta_0$$

Specific viscosity $-\eta_{sp}-$ Relative increase of viscosity

$$\eta_{sp} = (\eta - \eta_0)/\eta_0$$
$$= \eta_r - 1$$

η_{sp} is dependent upon the concentration and the ratio η_{sp}/c is defined as the viscosity number.

Extrapolation of viscosity number to infinite dilution yields the limiting viscosity number or intrinsic viscosity - $[\eta]$

$$[\eta] = \lim_{c \to 0} \eta_{sp}/c$$

The units of "c" used here are gm/ml as recommended by I.U.P.A.C. (J. Polymer Sci. 8 257, 1952).

Viscosity and Molecular Properties.

The problem of the correlation of viscosity with molecular dimensions has been approached in two ways. The first of these follows from the classical work of Einstein (1906) and attempts to treat the solute molecules as rigid, impermeable particles which may be approximated by spheres or ellipsoids (Simha, 1940). This method calculates the external dimensions of the solute particles from the viscosity without regard to the molecular configuration. The second approach deals with the more realistic model of coiled chain molecules which interact among themselves and with the solvent. The viscosity is derived by consideration of these interactions and of the distribution of chain elements in space.

The most useful result of the rigid particle approach is the equation of Simha (1940)

$$\eta_{sp}/\phi = \frac{J^2}{15(\ln 2J - 3/2)} + \frac{J^2}{5(\ln 2J - 1/2)} + \frac{4}{15} \qquad (1).$$

where J is the axial ratio of an ellipsoid and ϕ is the volume fraction of the solute.

The most serious drawback to the use of this equation, even in cases which are effectively rigid particles, is the need to know ϕ. This is the volume of the particle in solution, which may be quite different from the dry volume found by measurements of partial specific volume ($\bar{V}$). Thus the degree of solvation of the molecules must be determined if reliable results are to be obtained. Oncley (1941) has given calculations of the effect of solvation upon the apparent axial ratio for proteins.

The viscosity of real polymer molecules is, however, most often interpreted on the random coiled chain model which has been developed from the work of Kirkwood and Riseman (1948) by Flory and Fox (Flory, 1953). Using Einstein's equation the viscosity is related to an "equivalent hydrodynamic sphere"

$$[\eta] = 2.5\, N V_{(e)}/M \qquad (2).$$

N = Avogadro's No. V(e) = volume of equivalent hydrodynamic sphere.

By consideration of the distribution of chain elements and their interactions this volume (V(e)) is found to be proportional to the cube of the root-mean-square end-to-end length of the chain $(\overline{r^2})^{\frac{1}{2}}$

$$\text{Thus} \quad [\eta] = \Phi\, (\overline{r^2})^{3/2} /M \qquad (3).$$

where Φ is a universal constant, the best value of which is $2{\cdot}1 \times 10^{21}$

The theory shows that $(r^2)^{\frac{1}{2}}$ is dependent upon solvent / coil interactions and can be related to its unperturbed value $(\overline{r_o^2})^{1/2}$ by

$$(\overline{r^2})^{1/2} = \alpha\, (\overline{r_o^2})^{1/2} \qquad (4).$$

where α is known as the volume expansion factor.

If equations (3) and (4) are combined

$$[\eta] = K M^{1/2} \alpha^3 \qquad (5).$$

where $K = \Phi (\overline{r_o^2}/M)^{3/2}$ should be constant for a given polymer type.

An empirical equation of similar form (Mark 1938) has been widely used for expressing the dependence of viscosity on molecular weight.

$$[\eta] = K' M^{\beta}$$

where K' and β are constants only for a given polymer/solvent pair.

It must be emphasised that a dependence of $[\eta]$ upon molecular weight can only be expected for linear molecules. For compact, highly branched molecules, no such relationship will exist and, indeed, $[\eta]$ may be constant over a wide range of molecular weight.

Experimental.

The viscometers used in this work were of a modified Ubbelohde type [Ubbelohde (1937). Davis and Elliot (1949)].

In such a viscometer

$$\eta = K,\rho t - \beta,\rho/t$$

where ρ is density of solution, and β, is the kinetic energy factor necessary to account for the finite velocity of the liquid leaving the capillary. It is made negligible by having a long flow time, for $t > 200$ secs. in a typical viscometer β, may be ignored.

$$\therefore \quad \eta_{sh} = \rho t/\rho_o t_o - 1$$

and for dilute solutions $\rho = \rho_o$

$$\therefore \quad \eta_{sh} = t/t_o - 1$$

Method.

All solutions were carefully filtered through G3 or G4 sintered filters before use. The viscometer was held in a constant temperature bath at 22·5°. The minimum volume of solution necessary was slightly less than 10 ml. Two alternative procedures were employed, depending on whether the flow time for solvent was known.

(1) 10 ml. of solvent was placed in the viscometer and its flow time determined. Increments of polymer solutions (2 - 5 ml.) were added and the flow times found.

(2) 10 ml. polymer solution was placed in the viscometer and its flow time determined. 5 ml. additions of solvent were made and flow times measured.

Flow times were measured by stop-watch to the nearest 0·1 sec.

The first method was preferred as it provided a check on solvent flow time and gave better spacing of the concentrations.

The initial solution was made up by weight or, if this was not possible, its concentration was determined by hydrolysis to glucose and estimation of the glucose by the alkaline ferricyanide/ceric sulphate method [Lampitt, Fuller, Coton (1955)].

II D. SEDIMENTATION VELOCITY.

When particles move through a fluid under the influence of a gravitational or centrifugal field, their initially accelerated motion quickly reaches a terminal velocity whose value is determined by the frictional properties of the particles and the strength of the field. For particles of colloidal dimensions, fields of the order of 250,000 g. may be required. Ultracentrifuges capable of producing such fields have been designed by Svedberg and others (Svedberg and Pedersen, 1940).

Svedberg defined the sedimentation coefficient (S) as the velocity of sedimentation under unit centrifugal force.

$$\text{i.e.} \quad S = (dx/dt) \Big/ \omega^2 x \qquad \text{c.g.s. units}$$

$$= \left[(dx/dt) \Big/ \omega^2 x\right] \times 10^{13} \qquad \text{Svedberg units} \qquad (1)$$

where x = distance from centre of rotation (cms.)

ω = angular velocity (radians / sec.)

Sedimentation coefficients determined at temperatures other than 20° and in media other than pure water are usually corrected to this temperature and solvent.

$$S^{\circ}_{20} = S \; \frac{\eta \; (1 - \bar{v}^{\circ}_{20} \cdot \rho^{\circ}_{20})}{\eta^{\circ}_{20} \; (1 - \bar{v}\rho)} \qquad (2)$$

S°_{20} corresponds to η°_{20}, ρ°_{20}, the viscosity and density of pure water at 20°, and $\bar{v}^{\circ}_{20}$ is the partial specific volume of solute in this medium. Symbols without subscripts refer to experimental conditions (Svedberg and Pedersen, 1940).

Sedimentation and Molecular Weight.

Consider a homogeneous dispersion of particles in a centrifugal field $\omega^2 x$ at point x. The centrifugal force on a particle is balanced exactly by the frictional force, this being determined by the product of the terminal velocity and a frictional coefficient $f(s)$.

$$f(s) \cdot (dx/dt) = (M/N) \cdot (1 - \bar{v}\rho) \cdot \omega^2 x$$

where M = Molecular weight N = Avogadro's number.

$$M = \frac{N f(s) \cdot (dx/dt)}{(1 - \bar{v}\rho) \cdot \omega^2 x}$$

$$= \frac{F(s) \cdot S}{(1 - \bar{v}\rho)} \qquad (3)$$

It can be shown that the frictional coefficient is related to the diffusion coefficient D by

$$F(D) = R T / D$$

where R = gas constant

and hence, making the assumption that $F(s) = F(D)$

$$M = \frac{R T s}{(1 - \bar{v}\rho) D} \qquad (4)$$

This derivation makes no assumption of particle shape and, hence, should be universally true.

Sedimentation and Molecular Dimensions.

Measurements of sedimentation coefficient may be used to give some measure of the shape and dimensions of the solute molecules, as these are obviously related to the frictional coefficient.

From equation (3) the actual frictional constant can be calculated. This is then compared with that of a hypothetical spherical molecule of the same weight and density, calculated from Stokes's Law

$$F_0 = 6\pi\eta(3M\bar{v}N^2/4\pi)^{1/3} \qquad (5)$$

The ratio F/F_0 is called the frictional ratio and is a measure of the asymmetry of the molecule. However, increased F/F_0 also results from solvation and the effect of this factor must be eliminated.

The frictional ratio can be expressed as the product of two ratios - $F/F_0 = (F/F_s).(F_s/F_0)$

the first, F/F_s, being concerned with solvation and the second, F_s/F_0 with asymmetry.

If the amount of solvation is known, F / F_s can be calculated

$$F/F_s = (1+\omega/\bar{v}\rho)^{1/3} \qquad \text{(Kraemer, 1940)} \qquad (6)$$

The ratio F_s/F_0 can now be evaluated in terms of an ellipsoid of revolution (Perrin, 1936)

$$F_s/F_0 = \frac{(1-b^2/a^2)^{1/2}}{(b/a)^{2/3}.\ln\left[\frac{1+(1-b^2/a^2)^{1/2}}{b/a}\right]} \qquad (7)$$

where $b/a < 1$ is the axial ratio of a prolate ellipsoid.

This interpretation can be of considerable value where the approximation to a rigid ellipsoid is valid and where the degree of solvation can be measured. Where, however, the molecules are not rigid or have a high degree of solvation, it is better to use the method of Scheraga and Mandelkern (1953) which combines the results of sedimentation and viscosity measurements. By solution of simultaneous equations, the molecular volume can be eliminated and hence no knowledge of solvation is required.

Then $$\beta = NS [\eta]^{1/3} \eta_0 / M^{2/3}(1-\bar{v}\rho) \qquad (8)$$

where β is a factor related to the axial ratio of an ellipsoid and varying from (2-4) x 10^6 (the value of 2·1 x 10^6 corresponds to a spherical molecule). Having obtained the shape factor the "effective hydrodynamic volume" can be calculated, although this may not bear any direct relation to the actual molecular volume.

For random-coiled molecules, Mandelkern and Flory (1952) have derived an equation similar to (8) in which, however, β is replaced by a constant term $\Phi^{1/3} \cdot \rho^{-1} = 2{\cdot}11 \times 10^6$. This is derived from a statistical study of solute / solute and solute / solvent interaction. (The fact that $\Phi^{1/3} \cdot \rho^{-1}$ has the same value as β for spheres is not coincidental, but is because the "time-average" distribution of chain elements has spherical symmetry).

Concentration Dependence of Sedimentation Coefficient.

As is the case with all other solution properties of macromolecules, non-ideal behaviour occurs in solutions of appreciable concentration. This is due to interference among

the sedimenting molecules and is particularly important where long chain molecules are concerned. Due to this molecular entanglement, free sedimentation may not take place even at the lowest accessible concentration. To eliminate this effect, sedimentation coefficients obtained over a range of finite concentration are extrapolated to zero concentration. For many systems of interest, this dependence is linear, or nearly so. It has been found (Gralen, 1944) that the dependence can be expressed by an equation of the form

$$s = s_o/(1+kc)$$
$$= s_o(1-kc+k^2c^2-\cdots)$$

where terms in "c" higher than the first are seldom necessary. This empirical relation has been used in many forms, e.g. $1/s$ vs c or sc vs c . In addition some attempts have been made to derive a theoretical relationship (Fessler and Ogston, 1951).

For large, extended molecules, extreme curvature in the plot of S vs C is found. The least precise results are those measured at the lowest concentration and as it is these upon which the greatest weight is placed in extrapolation, the validity of the extrapolated value (S_o) is doubtful.

Experimental.

A Spinco Model E Analytical Ultracentrifuge was used in this work. The solution was contained in a cell consisting of a centrepiece with a sector-shaped compartment clamped between two quartz discs, and held in a cylindrical housing. The optical path

length was either 12 mm. or 30 mm.

For centrifugation the cell was placed in the rotor with the sector radially aligned. The rotor was freely suspended from the motor unit by a flexible shaft inside a steel vacuum chamber. The chamber was evacuated by an oil diffusion pump and a rotary oil pump. In most of the work recorded here, the temperature of the rotor was measured at the beginning and end of the run by a contact thermocouple. At a later stage, a Rotor Temperature Indicating and Control Unit became available, by means of which the rotor temperature could be controlled to $\pm 0.02^{\circ}$ during spinning. Sedimentation boundaries were observed and recorded photographically with a Philpot Svensson optical system. This produces a record of the variation of refractive index gradient throughout the cell.

Calculation of sedimentation coefficients.

Photographs of the boundary were taken at intervals during centrifugation. The distance from the point of maximum refractive index gradient to a fixed reference line was measured to 0·01 mm. with a two-dimensional travelling microscope. This was corrected for the magnification of the optical system M(= 2·215) and by addition of the known position of the reference line, the distance from the centre of rotation (x) was obtained. Log x was then plotted against "t" and S calculated from an integrated form of equation (1)

$$S = (2{\cdot}303 / \omega^2) (d \log x / dt)$$

(As repetition of this series of arithmetical manipulations was found to be tedious and time-consuming, a table was constructed which gave directly the value of log x for a distance on the sedimentation diagram. This device reduced the time involved in calculation by about a half).

II E.

PARTIAL SPECIFIC VOLUME.

The calculation of molecular weight from sedimentation coefficient measurements requires a knowledge of the density of the polymer molecules. While this, strictly, should be the actual density in solution of the possibly solvated molecules, it has been shown (Kraemer, 1940 b) that the density of the dry polymer is a sufficiently good approximation.

The term $\bar{V}$, appearing in the Svedberg equation $\left[M = RTS / D(1-\bar{V}\rho) \right]$ is the reciprocal of the density - the partial specific volume. This can be defined as the increase in volume of a solution when 1 gm. of solute is added to a large volume.

For a two component system, the partial specific volume of component (1), $\bar{V}_1$, is given by

$$\bar{V}_1 = (\delta v/\delta m_1)_{m_2} \qquad (1)$$

where v is the volume of solution and m_1, m_2 are the weights of the two components.

The specific volume of solution is

$$V = v/(m_1+m_2)$$

and

$$v = V(m_1 + m_2) \qquad (2)$$

Hence

$$\bar{V}_1 = (\delta v/\delta m_1)_{m_2} = V + (m_1+ m_2)\, dV/dm_1$$

Now the weight fraction of 2 is

$$W_2 = m_2 /(m_1 + m_2)$$

and hence $d W_2 = - m_2 d m_1 / (m_1 + m_2)^2$ (when m_2 is constant)

$$= - W_2 \, d m_1 / (m_1 + m_2) \qquad (3)$$

Combining (2) and (3)

$$\bar{V}_1 = V - W_2 \, d V / d W_2 \qquad (4)$$

This equation can be expressed in a more convenient form in terms of the density ρ of the solution.

For

and therefore $dV/dW_2 = -(1/\rho^2).(d\rho/dW_2)$

Also $W_2 = 1 - W_1$

$$\therefore \quad \bar{V}_1 = V + \frac{(1-W_2)}{\rho^2} \cdot \frac{d\rho}{dW_2}$$

$$= \frac{1}{\rho}\left(1 - \frac{(1-W_2)}{\rho} \cdot \frac{d\rho}{dW_1}\right)$$

$$\therefore \quad (1 - \bar{V}_1\rho) = \frac{(1-W_1)}{\rho} \cdot \frac{d\rho}{dW_1} \qquad (5)$$

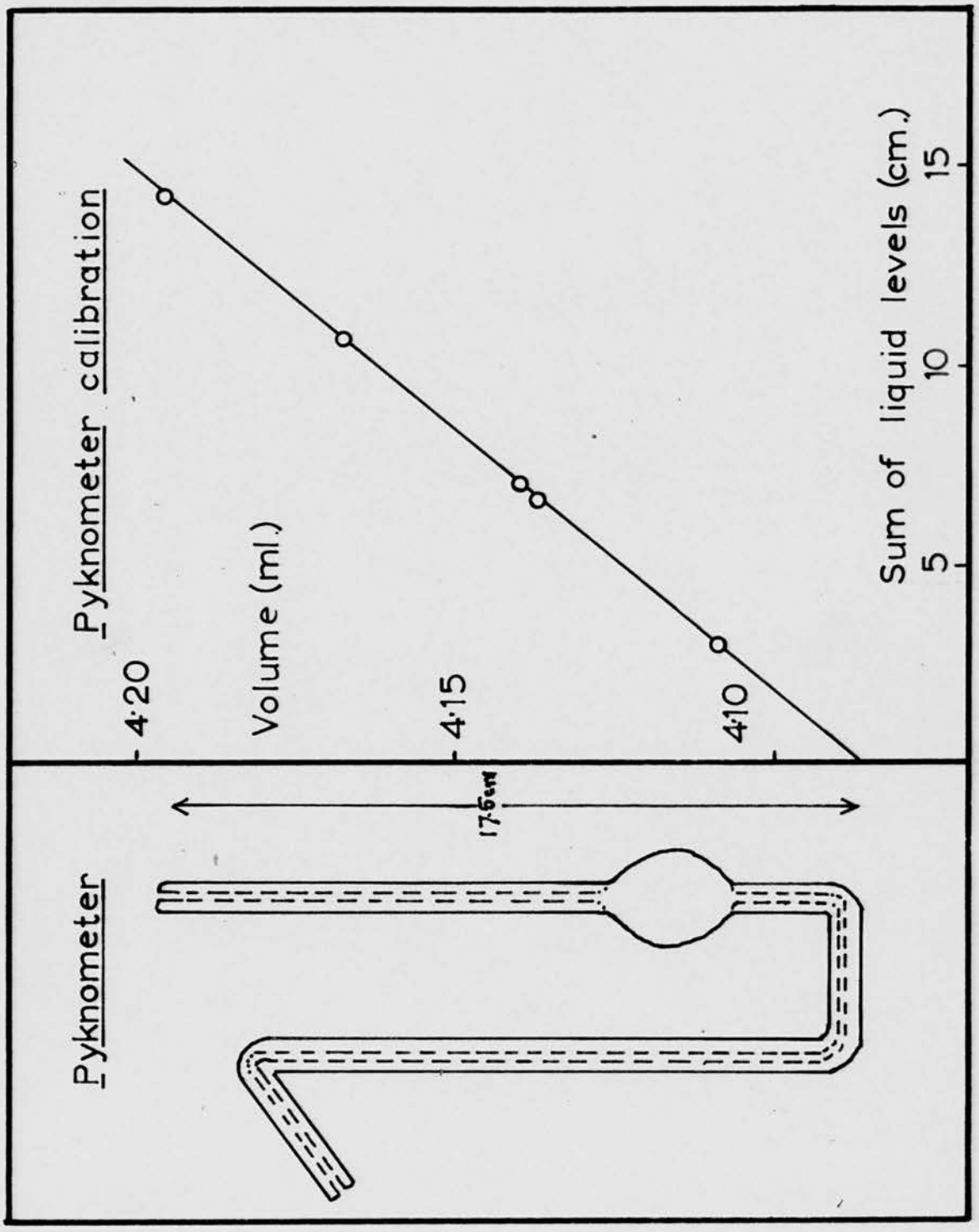
Using equation (5), $(1 - \bar{V}_1\rho)$ can be calculated from the densities of a series of polymer solutions of known concentration.

Measurement of density of solutions by pyknometry.

Density was determined in a pyknometer of design similar to that of Lipkin, Davidson, Harvey and Kurtz (1944) (Fig. 9) The capillary was of precision bore (0·6 mm.) and the volume of the bulb approximately 4 ml.

The pyknometer was filled by dipping the bent arm into the

FIG. 9


Pyknometer
Pyknometer calibration
4·20
4·15
4·10
Volume (ml.)
17·6cm
Sum of liquid levels (cm.)
5
10
15

liquid which was drawn into the bulb, first by capillary action and then by siphoning. The volume of liquid was sufficient to ensure that the levels in each arm were above the etched marks. The apparatus was placed in a constant temperature bath at 22·5° and allowed to come to temperature equilibrium. The heights of the liquid levels above the marks were measured to the nearest 0 001 cm. with a cathetometer. The pyknometer was removed from the bath, dried by a standardised procedure and weighed (to 0·01 mg.). The weight of the liquid was then obtained by difference. The volume of the apparatus was related to the height above the marks by the following calibration procedure.

Calibration - A. R. benzene was used to calibrate the pyknometer. Various volumes of benzene were drawn into the apparatus and the weights, and heights above the standard marks, measured. Knowing the weight and density of benzene, the actual volume was calculated. This was plotted against the sum of the heights of the liquid levels. (Fig. 9)

Sum of levels - S cm.	Wt. of benzene - gm.	Volume benzene - V ml.
2·856	3·59965	4·10894
3·053	3·60044	4·10984
6·577	3·62424	4·13701
6·979	3·62657	4·13967
10·590	3·65114	4·16772
14·210	6·67527	4·19525

Density of benzene at 22·52° = 0·87605

These data were evaluated by the method of least squares, giving

$$V = 4{\cdot}08677 + 0{\cdot}00763S$$

Using this equation, the volume can be obtained from the sum of the liquid levels.

Measurement of Density with density gradient columns.

Some preliminary experiments were made to investigate the possibility of using density gradient columns to determine density. This would be useful in that only very small quantities of polymer solution are required. Such a method also would be much more rapid and simple in use than conventional pyknometry.

It was shown by Linderstrom-Lang and Lanz (1938) that if two liquids of differing density were layered over one another in a narrow tube and partially mixed, a "density gradient" was set up which might be stable for many weeks. If a drop of an inmiscible liquid was then added to the tube, it floated at a level determined by its density. When carefully prepared, such a density gradient was sufficiently uniform to allow the determination of density by reference to standard solutions of known density. An accuracy in density determination of about 1×10^{-5} gm. /ml. was attained in these early experiments. However, this method of preparing density gradients was not reproducible, and the gradient was not completely linear.

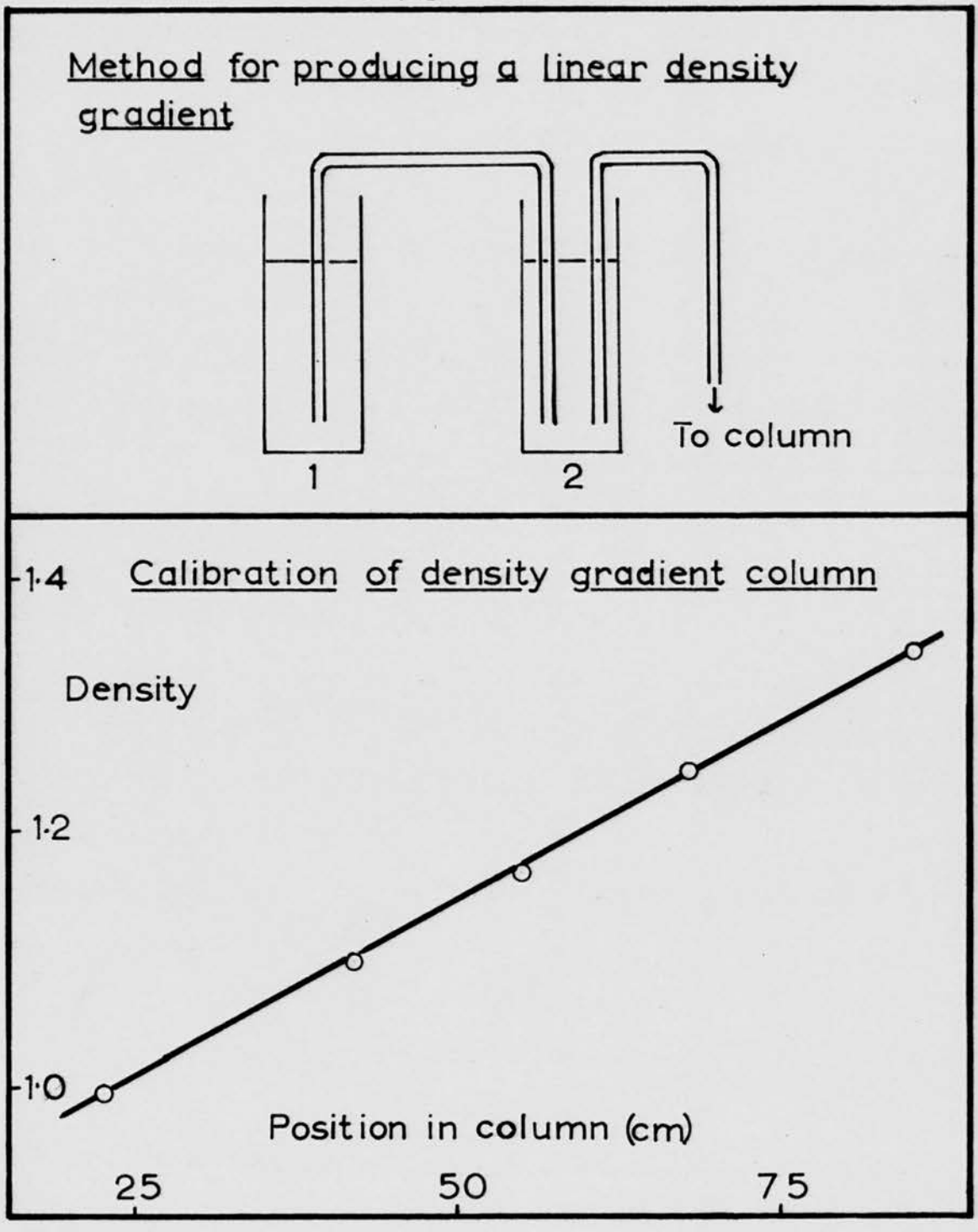
Recently, several attempts have been made to produce linear gradients by mechanical means. Mills (1956) used a system of

motor driven reservoirs. Tung and Taylor (1956) used a much simpler method, which is shown diagrammatically in Fig. 10 . The two starting liquids are contained in vessels (1) and (2) of the same cross section - the less dense liquid being in (1). The two vessels are connected by a siphon and an efficient stirrer mixes the liquids in (2). A siphon transfers the mixture from (2) into the long narrow tube (3), in which the gradient is set up.

It is easily shown that, provided the levels in the two vessels follow one another closely, a linear gradient is produced. However, with the apparatus as described, a difference in the two levels was immediately set up when using liquids of appreciably different density. This was due to the density difference between liquid inside the arm of the siphon and in vessel (2). This head decreased as the density in (2) was gradually lowered and, hence, a linear gradient was not produced. In order to avoid this difficulty it was necessary that the arm of the siphon in (2) should be only slightly below the liquid surface at all times. This was arranged by fitting a slightly larger sliding glass tube round the siphon tube, with a mercury seal at the top. As the level of the liquid in (2) fell, the outer tube was lowered gradually, so that its tip was always about 1 cm. below the surface.

The two vessels were held in a constant temperature bath and water from this bath was circulated through the jacket of the gradient column. This column was 90 cm. long and 2 cm. internal diameter.

FIG. 10


Method for producing a linear density gradient
To column
1
2
1·4
Calibration of density gradient column
Density
1·2
1·0
Position in column (cm)
25
50
75

A density gradient was prepared from carbon tetrachloride and benzene, covering the range 1·00 — 1·40 gm./ml. It was calibrated using standard solutions of potassium iodide. The gradient was linear over the range (see Fig. 10) and was stable for several days. It was used in the work described in Section III E.

To measure densities of polymer solutions in the range 0 - 1% concentration, a gradient covering density from 1·001 — 1·006 is required and for best accuracy this should be spread out over as great a length as possible. Attempts were made to prepare suitable gradients using the apparatus described with various liquid combinations e.g. carbon tetrachloride / hexane and mono-chlorobenzene / tetrahydro naphthalene. Appropriate mixtures of these liquids covering the desired range were used to prepare the columns. While these gradients were approximately linear, the uniformity and stability were low, and hence the required accuracy could not be attained. Thus, while a gradient of 10^{-4} gm./ml.cm. was easily prepared, it was found that a group of identical drops usually covered almost 1 cm.

It is thought that this poor uniformity was due to insufficiently good temperature control and to mechanical vibration. Better performances could probably be obtained by mounting the tube within a large constant temperature bath. A carefully designed shock-proof mounting might also be required. However, this was not further investigated.

II F.

EVALUATION OF THE DISTRIBUTION OF MOLECULAR WEIGHT.

At the present time, most - if not all - macromolecular compounds have been shown to be heterogeneous with respect to molecular weight. For such a system, any method of determining molecular weight must give an average value. The type of average obtained depends upon the characteristics of the method. Thus, colligative methods (osmotic pressure, vapour pressure) are sensitive to the actual number of molecules present but not to their size. These methods, therefore, yield a simple number-average molecular weight ($\bar{M}_N$) given by

$$\bar{M}_N = (\Sigma n_i M_i)/\Sigma n_i$$

where there are n_i molecules of molecular weight M_i.

NOTE.

The following terms will be used to describe the types of molecular weight distribution observed.

(1) Mono-molecular - a material completely homogeneous with respect to both composition and molecular weight.

(2) Poly-molecular - a material of uniform composition but with a continuous range of molecular weights.

(3) Poly-disperse - a material in which the molecular weights fall into two or more distinct ranges which may also differ in composition.

Light scattering, on the other hand, is sensitive both to the concentration (c_i) and weight of the molecules and hence a weight-average molecular weight ($\overline{M}_w$) is obtained, where

$$\overline{M}_w = \left(\Sigma c_i M_i\right) \Big/ \Sigma c_i$$

$$= \left(\Sigma n_i M_i^2\right) \Big/ \left(\Sigma n_i M_i\right)$$

The other common methods of determining molecular weight, viscosity and sedimentation and diffusion yield poorly defined averages which are dependent upon the hydrodynamic form of the molecules (see section III B,p.95).

In the study of degradation reactions of polymers, the change of molecular weight has often been followed. However, neither number- nor weight-average methods are ideal for this purpose. Changes in the number-average molecular weight merely indicate the number of bonds broken throughout the reaction mixture and no distinction can be made between a reaction splitting off monomer units from the polymer and one in which bonds near the centre of the molecules are broken. The weight-average molecular weight, on the other hand, would be almost unchanged by a reaction of the first type but would be particularly sensitive to the second type. Neither method, then, is of much value alone, although some useful information might be obtained from a study of changes in the ratio $\overline{M}_w / \overline{M}_N$ during the course of a reaction.

For the complete evaluation of changes in molecular weight during degradation, it is clear that measurement of the distribution of molecular weight throughout the sample would be desirable.

The various methods which have been applied include -

(1) Fractionation of the polymer by various methods, usually dependent upon differences in the solubility of the different molecular weight species.

(2) Frontal analysis of effluents from chromatographic separations (Claesson, 1949).

(3) Turbidimetric titration.

(4) Analysis of sedimentation equilibrium data (Svedberg & Pederson, 1940).

(5) Analysis of the spreading of the boundary obtained in sedimentation velocity experiments in the ultracentrifuge.

Methods (1) and (4) give good results but are very time-consuming. Methods (2) and (3) are more rapid but are difficult to make absolute. The fifth method is the most direct andwwill be described in detail below. An earlier example of this method was that developed by Gralen (1944) in which the width (B) of the sedimentation boundary gradient curve is defined as the ratio of area to maximum height. B is plotted as a function of the position (x) of the sedimentation boundary and the slope dB/dx used as a qualitative measure of polymolecularity.

ANALYSIS OF SEDIMENTATION VELOCITY BOUNDARIES.

(1) THEORY.

In the ideal case of a non-diffusing, mono-molecular substance, the boundary produced in the velocity ultracentrifuge would be infinitely thin. In practice, however, spreading of the boundary

occurs as a result of heterogeneity in molecular weight and of diffusion.

Consider first the effect of heterogeneity alone with the additional simplification that sedimentation rate is independent of concentration.

The patterns produced by the Schlieren optical system of the ultracentrifuge measure directly the variation of refractive index gradient (dn/dx) with distance from the centre of rotation (x). A knowledge of the optical factors allows the conversion of (dn/dx) into (dc/dx).

The polymer solution may be considered to consist of species 1, 2, 3.....n, with sedimentation coefficients S1, S2, S3.....Sn. Each species (i) sediments independently, forming a sharp boundary at x_i. Thus at plane x_i species i changes in concentration from zero to c_i. Species of lower sedimentation coefficient are also present at this boundary but are of constant concentration. The concentration (c_i) of i must then be given by $(dc/dx)_i$.
A distribution function g(x) can then be defined as

$$g(x) = (1/c_o)\ (dc/dx)_i \qquad (1)$$

where c_o is the total concentration.
A distribution according to x is thus obtained.

The more useful distribution would be according to S. This can be calculated by converting x values into S values by

$$S_i = \ln(x/x_o) \ / \ w^2t \qquad (2)$$

where x_o corresponds to $t = o$.

The corresponding distribution function g(S) is given by

$$\begin{aligned} g(S) &= dc/ds \\ &= (dc/dx)\ (dx/ds) \\ &= g(x)\ \ xw^2t \end{aligned} \qquad (3)$$

However, $(dc/dx)_i$ does not truly represent the change in concentration at plane i because of two factors which combine to give a "dilution" of the boundary with increase in x. These are the sectorial shape of the cell and the fact that the centrifugal field is proportional to x. Svedberg and Rinde (1924) showed that these effects are expressed by

$$(dc_o/dx) = (dc/dx)\ (x/x_o)^2 \qquad (4)$$

where c_o is the actual concentration

g(S) is now given by

$$g\ (S) = (dc/dx)\ (x/x_o)^2\ (xw^2t/c_o) \qquad (5)$$

This equation was obtained by Signer and Gross (1934) and is valid only when diffusion may be neglected. Diffusion also produces increased spreading with time and hence curves of g(S) vs S obtained at different times after sedimentation starts are not identical. Baldwin and Williams (1950) have shown that it is possible to eliminate the effect of diffusion since, while the spreading due to heterogeneity is proportional to the time (t), that due to diffusion is proportional only to the square root of the time ($t^{\frac{1}{2}}$). Thus the relative contribution of diffusion to the observed spread of boundary

decreases with time and becomes negligible at infinite time. An extrapolation of values of g(S) for fixed values of S against 1/xt to 1/xt = 0, has been shown (Gosting, 1952) to yield a distribution from which the effect of diffusion has been eliminated.

It is, therefore, possible to obtain the distribution of sedimentation coefficients g(S) vs S, corrected for diffusion, from several apparent distributions $g^*(S)$ vs S obtained at suitable time intervals during sedimentation.

The foregoing treatment has been for the case of concentration independent sedimentation. All systems of interest show a decrease of sedimentation coefficient with increasing concentration. This results in a modification of the shape of the sedimentation boundary. Two distinct causes are recognised, (a) boundary sharpening and (b) the Johnston-Ogston effect (Johnston and Ogston, 1946).

(a) The concentration of solute increases from zero to its final value across the width of the boundary. Thus the sedimentation rate of the slowest species is virtually unmodified by the low concentration at the trailing edge of the boundary, while the rate of the fastest species is considerably reduced by the much higher concentration at the leading edge. A marked narrowing of the boundary therefore results.

(b) Johnston and Ogston (1946) have shown, in the simple case of a mixture of a fast and a slow component, that the apparent concentration of each is modified by the presence of the other. The apparent concentration of the slow is larger and of the fast

component, smaller than the actual concentration, as a consequence of concentration dependence. They pointed out that, because of the increased concentration, the rate of the slower component in front of the fast boundary was slower than behind it. Thus the concentration of the slow component tends to build up behind the fast boundary to a level higher than in the bulk of the solution. A decrease in the concentration of the slow component takes place across the fast boundary and hence the apparent concentration of the fast component is less than its bulk value. A similar situation must exist in the sedimenting boundary of a polymolecular material. The value of $(dc/dx)_i$ does not truly represent the concentration of species i, as was formerly assumed, but is less than this because of the decrease in concentration of the slower species occurring at plane i. Thus it is only in the solution beyond the boundary - "the plateau region" - that the concentrations of the species attain their true relative proportions.

To obtain absolute distributions of sedimentation coefficients these concentration effects must be corrected for. This correction may be either by a graphical or by an analytical procedure.

In the graphical method (Williams and Saunders, 1954), (Erikson, 1956), distributions, corrected for diffusion, are obtained at a series of concentrations. Values of g(S) for fixed values of S are then plotted as a function of c and $g(S)_o$ obtained by extrapolation.

Baldwin (1954) has made correction for concentration effects by an analytical procedure. This involves the determination of a distribution at one concentration and a knowledge of the concentration dependence of S. The corrections for Johnston-Ogston and boundary sharpening are made separately.

(i) Johnston-Ogston effect. Baldwin showed that the change in concentration $(\Delta c_i)_j$ of species i at plane j, is related to the change in its sedimentation coefficient ΔS_i. ΔS_i is determined by the change in total concentration between planes i and j.

$$\text{Then} \qquad (\Delta c_i)_j = \frac{c_i \cdot \Delta S_i}{\left[\ln (x_j/x_o)/\omega^2 t - (S_i)_j \right]}$$

$$= \frac{c_i \cdot \Delta S_i}{S_j - (S_i)_j} \qquad (6)$$

where c_i is the concentration of i at the previous plane and $(S_i)_j$ its sedimentation coefficient at plane j.

Using this relation the concentration of each species can be found in the "plateau region".

(ii) Boundary sharpening. This correction involves the conversion of the distribution g(S) vs S into $g(S_o)$ vs S_o.

$$\text{As} \qquad g(S_o) = dc/dS_o$$

$$= (dc/dS)\,(dS/dS_o) \qquad (7)$$

$$= g(S).\,dS/dS_o$$

it is only necessary to determine dS/dS_o from the concentration

dependence at each plane throughout the boundary and plot $g(S_o)$ against the corresponding S_o.

By these correction procedures, then, an absolute distribution of sedimentation coefficients can be obtained. The final stage in the determination of molecular weight distribution requires a knowledge of the relationship between molecular weight and sedimentation coefficient derived from a study of a series of fractions of differing molecular weight. Such a relation is probably the least reliable stage in the determination of distributions as, unless very sharply fractionated samples are available, the exact significance of the molecular weight obtained from sedimentation is doubtful (See section III B). The actual method of conversion is quite simple since

$$\begin{aligned} g(M) &= dc/dM \\ &= (dc/dS)_o \ (dS_o/dM) \\ &= g(S_o) \cdot dS_o/dM \end{aligned} \qquad (8)$$

This sequence of operations allows the determination of the distribution of mass over the range of molecular weight. There are two points at which errors may be produced. These are the forms of the relation between S and c and between S and M as indicated above. The dependence of S upon the total concentration only can be measured. It is, however, quite probable that S also depends upon the composition and that therefore the dependence for a complete sample will be different from that of a component of a

polymolecular mixture. However, it is not possible to measure the dependence upon composition and hence this effect must be neglected. Thus the absolute accuracy of molecular weight distribution is doubtful but it is believed that, for a series of similar samples, the relative values will be correct.

(2) Experimental.

(a) Measurement of the refractive index gradient curve - Photographs of the sedimenting boundary were taken with the Philpot-Svensson optical system. It was found that better defined photographs were obtained by using an inclined bar rather than a wire. When the wire was used, the strong interference fringes caused greater confusion of the image.

In order to measure the height - h - of the gradient curve it is necessary to have a "base-line" from which to refer measurements. In experiments at slow speeds the base-line is usually horizontal and can be fitted by merely producing the horizontal parts of the gradient curve on either side of the boundary.

Since a measured height - h - is related to dn/dx by

$$dn/dx = h \tan\theta / m_1 m_2 H_1 H_2$$

where θ is the angle of the bar, m_1, m_2, H_1 are constants of the optical system and H_2 is the thickness of the solution column, it follows that the concentration is

$$c = \left[\int (x/x_0)^2 . (dn/dx) . dx\right] / \Delta n$$

where Δn is the specific refractive increment.

The concentration calculated from this relation agreed with the actual concentration within about 10% in all cases.

Some measurements were made directly from the photographic plates with a two-dimensional travelling microscope. The reproducibility attained by this method was low, however, and it was found to be preferable to make photographic enlargements (9 diameters), which were then traced on to graph paper.

(b) Calculation of apparent distribution $g^*(S)$ - Values of h at 20 equally spaced intervals across the boundary were measured. Hence $g^*(S)$ was calculated from equation (5) using h for dc/dx and $(\Delta x \Sigma h(x/x_0)^2$ for c_0. Absolute values for these quantities are unnecessary since the proportionality factors disappear. Values of S_i were calculated from x_i using equation (2) and were corrected for the temperature of the experiment if this differed 20° (See Section II D equ. (2)).

(c) Elimination of diffusion - Apparent distributions were calculated from each of four or five photographs at different sedimentation times . Values of $g^*(S)$ at discrete values of S_i were plotted against $1/x_i t$. A linear extrapolation to $1/xt = 0$ was made for each and the extrapolated value $g'(S)$ was plotted against S to give a distribution corrected for diffusion.

The area under the curve was found to have remained almost constant but, if necessary, a small correction of 5% or less was made to normalise the area.

(d) Before the Johnston-Ogston corrections can be made, the g'(S) vs S distribution must be converted into one of dc/dx vs x. The reason for this is that the concentrations used in the calculations must be those actually existing in the cell during the experiment.

$$\text{Thus} \qquad dc/dx = g'(S) \cdot x_o^2 \cdot c_o / x^3 w^2 t$$

Here t is the average time and c_o is the actual concentration of the solution.

(e) Correction for Johnston-Ogston effect - This correction involves the use of equation (6)

$$\text{i.e.} \qquad (\Delta c_i)_j = \frac{c_i \cdot \Delta S_i}{S_j - (S_i)_j}$$

The complexity of the arithmetical manipulations necessary depends strongly upon the form of the dependence of S on c. A relationship of the type

$$S = S_o - kc$$

would be ideally simple in use but was not found to be applicable to the materials used in this work. The relationship used was

$$S = S_o (1 - kc)$$

Hence

$$\Delta S = - kS_o \cdot \Delta c$$

The curve of dc/dx vs x was divided into some 20 equi-spaced lamellae each to be regarded as a separate species.

At plane (1) only species (1) is present and hence

$$c_1 = \Delta x \, (dc/dx)_1$$

At plane (2) the total concentration has increased by $\Delta x(dc/dx)_2$ which is the apparent concentration of species (2)

S_1 changes by

$$(\Delta S_1)_2 = - k (S_o)_1 \, \Delta x \, (dc/dx)_2$$

where $(S_o)_1$ is calculated from

$$S_1 = (S_o)_1 (1 - kc_1)$$

Thus the change in concentration of (1) at plane (2)

$$(\Delta c_1)_2 = c_1 (\Delta S_1)_2 \; / \; (S_2 - S_1 + (\Delta S_1)_2)$$

This value is negative and hence the actual value of c_2 is greater than $\Delta x \, (dc/dx)_2$ by this amount.

At the third plane the corrected values of c_1 and c_2 were similarly used to obtain the true concentrations of (1), (2) and (3) at plane (3).

The calculation was repeated for each plane until the corrected value of the concentration of each component in the "plateau region" was known.

(f) Correction for boundary sharpening. - The previous stage involved the calculation of S_o for each plane. Values of S_i and $(S_o)_i$ were tabulated and $(dS/dS_o)_i$ obtained by simple tabular differentiation.

Hence, using equation (7) the true distribution of sedimentation coefficient was calculated.

SECTION III.

STUDIES ON GLYCOGEN.

III A.

The Molecular Weight of Glycogens from Various Sources.

Glycogen is the reserve carbohydrate of all animals. As such, a semi-permanent store is maintained in the liver, while a more transient supply exists in muscle tissue. It is also found in many micro-organisms. Because of its important biological function, much work has been carried out on glycogen by both chemical and physico-chemical methods.

Glycogens from many sources have been examined and, apart from minor differences in chain length, all appear to have very similar chemical structure. However, the molecular weight of glycogen appears to vary with the source and, in general, glycogens from muscle tissue are smaller than those from the corresponding liver tissue.

Molecular weight determinations on glycogen may be made by most of the usual methods. From most sources, glycogen is obtained in a form which is readily soluble in aqueous systems to give a stable solution. There is, therefore, no need to resort to the preparation of derivatives, as is frequently the case for other polysaccharides.

The earliest reported molecular weights for glycogen were obtained by measurements of osmotic pressure [Oakley and Young (1936)] The values of $(0{\cdot}7 - 2{\cdot}2) \times 10^6$ established the highly polymeric nature of glycogen. These results may be regarded as a minimum value, as the osmotic pressure method is at the limit of its sensitivity in this molecular weight range, particularly in aqueous solution. Substantially similar results have been obtained by

Staudinger and Husemann (1937), Carter and Record (1939), Bell et al (1948) and Kerr et al (1951).

Sedimentation and diffusion measurements yield rather larger values, as would be expected for such a polymolecular material. Bridgman (1942) found 4×10^6 for the molecular weight of rabbit liver glycogen and showed that a very wide distribution of molecular sizes was present. Similar results were obtained by Bell et al (1948). More recent work by Polglase, Brown and Smith (1953) has suggested that glycogen may be polydisperse. Two components were found in some liver glycogen samples, the minor component having a sedimentation coefficient of up to three times that of the major component.

Light scattering has also been employed to measure the molecular weight of glycogen. The sizes indicated by this method are rather larger than by any other method. Thus, Putzeys and Verhoeven (1949) found weight-average molecular weights in the range $(6 - 18) \times 10^6$ for rabbit liver and muscle glycogen. It would appear that light scattering is probably the best method for such a highly polymolecular material as a true weight-average molecular weight is obtained, in contrast to the rather ill-defined average from sedimentation and diffusion measurements. However, light scattering can give no indication of polydispersity or even of degree of polymolecularity. These can be obtained readily from measurements in the ultracentrifuge and so, in order to obtain a true picture of molecular size and its distribution, it is desirable to make both light scattering and sedimentation measurements.

Such measurements are reported here on a range of glycogen samples. Also included are measurements of a polysaccharide obtained from sweet corn (Zea Mays) which appears to have all the chemical and structural characteristics of a glycogen.

EXPERIMENTAL.

Glycogen Samples. (kindly provided by Dr. D.J. Manners).

All glycogens were prepared by the classical Pfluger method (see p. 90). The commercial samples were from British Drug Houses (I) and Nutritional Biochemicals Corporation (II). The sweet corn glycogens were prepared by Dr. P.C. Das Gupta, [Greenwood and Das Gupta (1958)] and by Peat, Whelan and Turvey (1956).

Sedimentation measurements.

Measurements were made as described in Section II D. The freedom of the glycogens from electrolyte impurities was not certain and therefore most measurements were made in sodium chloride solutions of either 0·1 or 1·0 molarity. In some experiments 0·2 M potassium hydroxide was used. The sedimentation coefficients were corrected in the appropriate manner to the viscosity and density of water at 20° (p. 49). In most cases, measurements were made at four or more concentrations below 1%.

When a glycogen was definitely polydisperse, the apparent amount of each component was estimated from measurements of the areas under the sedimentation boundary. A tracing of the sedimentation diagram was made by projecting an image, enlarged three times, on to smooth

paper. The upper outline was traced. It was impossible to fit a base-line to the diagram by the usual method of producing the horizontal parts of the image on either side of the peak, as these were frequently distorted by the minor components, and it was uncertain where the image became truly horizontal. Therefore, comparative runs were made, under identical conditions, with solvent alone in the cell. The resulting image was then carefully aligned with that of the sedimenting solution and the base-line superimposed. The gradient curves were divided into components by assuming that each had a symmetrical distribution. The areas were measured with a planimeter.

Diffusion measurements.

These were made by Dr. W.A.J. Bryce in an Antweiler micro-electrophoresis and diffusion apparatus. The solvent was 0·1 M sodium chloride at 20° and diffusion coefficients were evaluated by the moment method.

Partial Specific Volume.

This was measured as described in Section II E. The partial specific volume of Commercial II glycogen in 0·1 M sodium chloride was found to be 0·62.

Refractive Index Increment.

Measurements were made as described in Section II B, on two samples of glycogen and one of sweet corn polysaccharide. The solvent was 0·1M sodium chloride and the light was of wavelength 546 mμ. The value of 0·1460 ± 0·0002 was found.

Light scattering measurements.

The Rayleigh ratio and dissymmetry were measured in a cylindrical cell. The light was the Hg green line, at 546 mμ. Solvents were water, 0·1M sodium chloride or 15% magnesium chloride. Clean solvent was obtained by filtration under gravity through G5 sintered Pyrex filters. Various other types of filter were tested. These included "Millipore" (type HA), "Ultrafeinfilter", and sintered copper sheet. Results with these filters were in no case better than with sintered glass, although the first two gave similar results. As these methods were much less convenient, sintered glass filters were used routinely. After careful washing, a new G5 filter gave reproducible dissymmetries of the order of 1·5 for water and 1·2 for salt solutions. After a period of use, however, the results deteriorated.

Solutions of glycogen were clarified by filtration through G4 sintered Pyrex. Preliminary experiments were made using G5 filters, but it was found that filtration was extremely slow, considerable amounts of glycogen were removed from solution, and no significant improvement in clarification was obtained. Millipore filters gave similar results. One filtration, under gravity, through G4 filters was found to be satisfactory, except where the solutions were very dusty. Repeated passage through G4 filters caused a small drop in turbidity, with little decrease in dissymmetry. This obviously indicated that removal of glycogen from solution was occurring, and hence, the concentration of

glycogen solutions was always measured after filtration, using the ceric sulphate/alkaline ferricyanide method.

The procedure finally adopted was to filter solvent directly into the clean cell. The amount of solvent was determined by weight. (1 - 2) ml. aliquots of the filtered solution (ca 0·1% concentration) were then added to give concentrations in the range of (1 - 20) x 10^{-5} gm/ml.

RESULTS AND DISCUSSION.

Effect of solvent composition.

TABLE I. Effect of solvent on Brewers' Yeast glycogen.

(a) Sedimentation $S_{20} \times 10^{13}$ at c (gm/100 ml.)

	1	0·75	0·5	0·25	0·16	0·125	0·08	S_o
in 0·1MNaCl	56	-	60	61	-	62	-	64
1·M NaCl	54							
0·2MNaOH	56	58	60	61	62	-	63	64

(b) Light scattering

Solvent	Water	0·1M NaCl	15% $MgCl_2$
$M \times 10^{-6}$	4·5	4·4	4·2

Sedimentation measurements were made on Brewers' Yeast glycogen in 0·1M, 1·0M sodium chloride and 0·2M sodium hydroxide. As shown in Table Ia, the sedimentation coefficients were the same in all three solvents, within the limits of experimental error. The concentration dependence was also unaltered.

15% Magnesium chloride solution has been recommended as a solvent for materials liable to aggregation in solution, as its high activity coefficient should make it an effective dispersing agent [(Staudinger (1948), Stetten et al (1956), Erlander and French (1958)] Light scattering measurements were made on Brewers' Yeast glycogen in 15% magnesium chloride, 0·1M sodium chloride and water (Table Ib). No difference was observed.

These results suggest that no aggregation is occurring and that the molecular weights measured are those of a truly molecular dispersion.

Concentration dependence.

Fig. 11 shows the relation between sedimentation coefficient and concentration for Brewers' Yeast and Commercial II glycogens, which are typical of those samples exhibiting no polydispersity. The results could be fitted by an equation

$$S = S_o (1 - kc)$$

where $k = 0{\cdot}10 \pm 0\ 02$ (see also section III B).

However, most of the samples examined were polydisperse to some extent and anomalous concentration dependence was observed. Thus, in some instances, S at the lowest concentration (0·2%) was actually less than at higher concentrations. This is partly attributable to experimental error, which is greatest at low concentrations, due to the spreading of the sedimentation boundary. However, apart from errors, the Johnston-Ogston effect (p. 69) at high concentration must cause some distortion of the relative proportions of the components and, hence, a shift of the maximum of the peak. It was not possible, however, to place too much significance upon the values found at low concentration because of their greater error and, therefore, S_o for such samples was either extrapolated from the other values or calculated from the results at one concentration using the relation given above.

FIG. 11

S vs C for

1 Brewer's Yeast glycogen

2 Commercial II .. ..

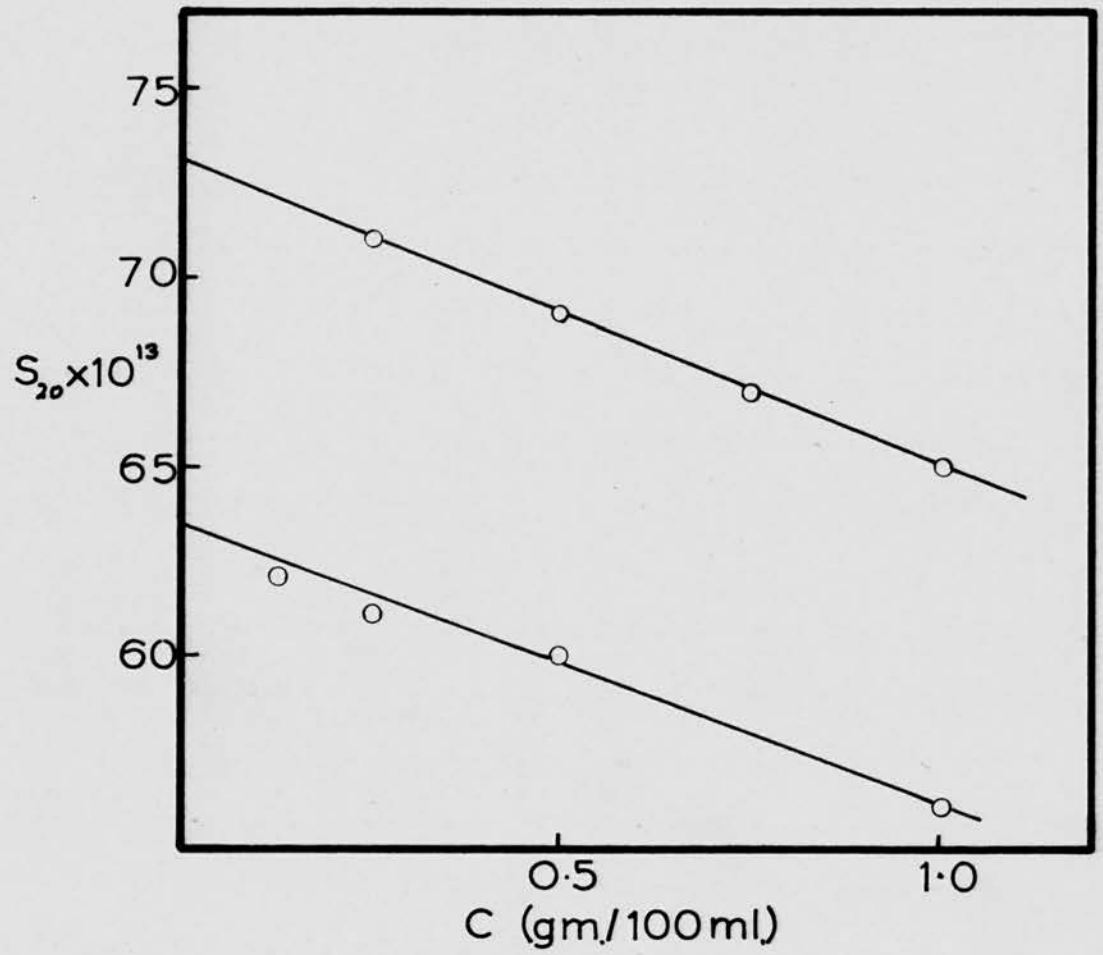


FIG. 12

Sedimentation patterns.

Speed = 20,000 r/m Concentration = 1gm/100ml.

Oyster glycogen

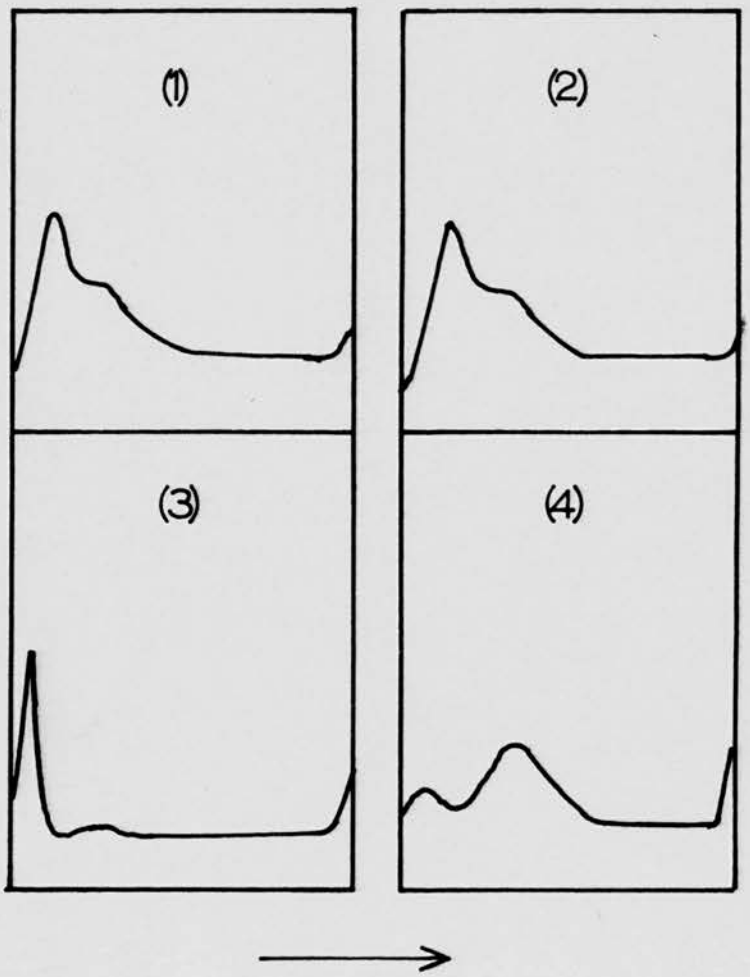
(1) 15min. $\angle = 45^\circ$

(2) 47min $\angle = 25^\circ$

Helix pomatia glycogen

(3) 6 min. $\angle = 65^\circ$

(4) 50 min. $\angle = 35^\circ$



Polydispersity.

The majority of the samples examined were polydisperse to some extent. Only Brewers' Yeast, Commercial I, II, Rabbit Liver II, Rabbit Muscle I and Methylated Horse Muscle glycogens appeared to be monodisperse. The sweet corn polysaccharides were also free from minor components. Even these materials showed a very wide but symmetrical distribution of sedimentation coefficients. Some samples were not obviously polydisperse but gave a very unsymmetrical sedimentation boundary. In some cases the high molecular weight side of the peak stretched almost to the bottom of the cell within a few minutes of the start of sedimentation. The converse, in which a probable slow component was present, was also observed. It was not possible to estimate the amount or sedimentation coefficient of such components and accordingly these are indicated in Table II with the symbols F or S respectively. Other samples were definitely polydisperse, and the sedimentation coefficient of the minor component could actually be estimated (Fig. 12). In Table II the apparent amounts of the major components of such samples are quoted. These are only approximate estimates since no attempt was made to correct the concentration for the Johnston-Ogston effect.

Molecular Weight.

Diffusion coefficients were determined for four samples.

	$D_m \times 10^7$
Methylated Horse Muscle	1·0
Brewers' Yeast	1·1
Commercial I	2·0
Commercial II	1·1

For other samples, molecular weights were calculated assuming a value of $D_m = 1{\cdot}1 \times 10^{-7}$ as an average applicable over most of the range.

These molecular weights are listed in Table II.

Molecular weights were obtained from light scattering measurements using the equation

$$\frac{Kc}{R_{90}} = \frac{1}{MP_{90}}$$

K had the value $1{\cdot}415 \times 10^{-7}$. At the concentration used $(1 - 20) \times 10^{-5}$ gm/ml., Kc/R_{90} was constant within the limits of error. An average of the values at the four concentrations measured was therefore taken. The particle scattering factor (P_{90}) was calculated from the dissymmetry, assuming that the molecules were spherical. Although it is unlikely that glycogen molecules are really spheres, this model gives the best approximation of those available. The molecular weights are given in Table III.

For the samples showing no polydispersity, the agreement of the molecular weights from the two methods is reasonable, although that from light scattering tends to be rather larger. This is to be expected since light scattering yields a true weight-average

TABLE II

Glycogen sample	S_{10} of components major	minor	Major Component (%)	10^{-6} M
Mammalian livers				
Cat I	75	F,S	-	4·4
" IV	84	F,S	-	4·9
" VI	(102)	F,S	-	5·9
Human (glycogen storage disease)	(53)	(220)	70	3·1
Foetal sheep	110		-	6·4
Foetal pig	(49)	(11)	70	2·9
Rabbit II	94	-	95+	5·5
" (fructose-infused)	(80)	F	-	4·7
" (galactose infused)	(153)	S	-	9·0
" (normal)	(145)	F	-	8·4
Mammalian muscle				
Methylated Horse	23	-	95+	2·8
Human	(85)	20	85	4·9
Rabbit I	79	-	95+	4·6
Other glycogens				
Ascaris lumbricoides	48	F	-	2·8
Brewers' Yeast	64	-	95+	3·7
Commercial I	24	-	95+	0·7
" II	73	-	95+	4·0
Helix pomatia II	(63)	(300,7)	80	3·6
Mytilus edulis I	(93)	F	-	5·4
Oyster	(45)	90,F	-	2·6
Tetrahymena pyriformis	(69)	S	-	4·0
Trichomonas foetus	(70)	S	-	4·0
Trichomonas gallinae	(84)	S	-	4·9
Sweet corn polysaccharide				
Phytoglycogen B	48	-	95+	4·0
Total polysaccharide	242	-	95+	2·9

TABLE III.

Sample	M x 10^{-6}	Dissymmetry	M_{ls}/M_{SD}
Liver glycogens			
Cat I	13·6	1·48	3·1
" IV	13·4	1·85	2·75
" VI	17·9	1·67	3·05
Rabbit II	7·8	1·20	1·4
Other glycogens			
Ascaris lumbricoides	8·9	1·40	3·2
Brewers' yeast	4·4	1·15	1·2
Commercial I	1·9	1·19	2·7
" II	5·4	1·15	1·35
Rabbit muscle I	4·1	1·17	0·9
Tetrahymena pyriformis	11·3	2·50	2·8
Sweet corn polysaccharide			
Phytoglycogen B	4·4	1·17	1·1
Total polysaccharide	2·7	1·35	0·95

molecular weight of all the molecules present. The molecular weight from sedimentation and diffusion does not strictly represent any average of the sample when evaluated from the movement of the maximum of the boundary gradient curve. At best, it represents the molecular weight of the component present in maximum amount. For a symmetrical distribution this might approach a weight average, but in nearly all the samples the distribution was skewed in the direction of high sedimentation coefficient and hence the weight average must inevitably be larger. This point will be discussed more fully in Section III B. The agreement for the two sweet corn polysaccharides is particularly good. This is a result of the extremely symmetrical distribution, especially of the total polysaccharide.

All other samples were polydisperse and hence the discrepancy between the two molecular weights is much greater. In fact, the occurrence of such discrepancies is a very sensitive test for the presence of large components.

III B.

Effect of Isolation Procedure on the Molecular Weight of Glycogen.

Glycogen can be extracted from tissue by three main methods. These involve solution of the glycogen in hot 30% aqueous sodium or potassium hydroxide, in hot water or in cold dilute trichloracetic acid. By far the greatest amount of work has been carried out on glycogens isolated by the alkaline extraction method, first employed by Pfluger (1903). In this method, the tissue is completely solubilised by heating in strong alkaline solution at 100° for about three hours. The possibility, that degradation may occur under such drastic conditions, seems to have been ignored by most workers. The alkaline degradation of polysaccharide materials is a well established phenomenon which appears to involve two mechanisms. The first is a random hydrolytic breakdown of glycosidic bonds; the second is a specific attack from the reducing end of the molecule with the stepwise elimination of mono-saccharinic acids (Whistler, 1958). It is, therefore, almost inevitable that glycogen, extracted by the Pfluger method, should be degraded. Extraction of glycogen with hot water leads to molecular sizes of the same order as does alkaline extraction (Greenwood and Manners, 1958). The reason for this is not known.

However, when glycogen is isolated after extraction of the tissue with ice-cold 10% trichloracetic acid, material of much greater apparent molecular weight - (50 - 100) x 10^6 - is obtained (Stetten et al, 1956). The molecular weight was determined by light scattering. An earlier investigation of this isolation procedure by ultracentrifugation (Bridgman, 1942), gave very

similar results to alkaline extraction. Bridgman, however, did not take any precaution to limit the period of exposure of the glycogen to acid and degradation probably occurred . On the other hand, it is possible that the divergence was merely an extreme case of the behaviour found in the previous section, and that the molecular weights obtained by Stetten et al were heavily weighted by small amounts of very high molecular weight components.

It was therefore of interest to examine glycogens, isolated by alkaline and acid extraction methods, by both ultracentrifugation and light scattering, in an attempt to determine whether the differences between them are real and, further, whether the acid extracted material is a molecular dispersion or merely aggregates.

EXPERIMENTAL.

Isolation of glycogen.

(1) Alkaline extraction.

Rabbit liver was finely minced and heated on a boiling water bath for three hours with 30% aqueous sodium hydroxide. At the end of this period the mixture was allowed to cool. The layer of soap, which then formed on top, was removed and the mixture centrifuged. The precipitated cell debris was re-extracted and the combined alkaline solutions filtered through glass wool. The addition of 1½ volumes of ethanol then precipitated crude glycogen. This was redissolved in water, centrifuged and reprecipitated with ethanol. The precipitate was again dissolved and centrifuged. Four volumes of glacial acetic acid was then added to precipitate the glycogen. This procedure was repeated twice and caused considerable improvement in the colour of the glycogen. In order to remove the brownish material which was still present, ethanol was added carefully to produce a faint turbidity. On centrifugation a gummy brown material was obtained and discarded. The pure glycogen remaining in solution was completely precipitated with ethanol, and dried by washing with ethanol and ether. This material is termed OH-glycogen.

(2) Trichloracetic acid extraction.

Minced rabbit liver was treated with 10% trichloracetic acid at 0° - 2° in a high speed macerator for 2 - 3 minutes. The mixture was filtered through muslin and the glycogen immediately

precipitated from the filtrate by the addition of 1½ volumes of ice-cold ethanol. The precipitate was collected by centrifugation, also at 0° - 2°. Meanwhile, the residue from the first extraction was re-extracted with cold 5% trichloracetic acid and the glycogen combined with the first precipitate. The crude glycogen was redissolved, centrifuged and precipitated with ethanol. This process was repeated until the addition of a trace of lithium bromide was required to induce flocculation on the addition of ethanol. The purified glycogen was then dried as before, This material is termed TCA -glycogen.

Usually these two isolation procedures were carried out in parallel on halves of the same rabbit liver.

Purification by the second method was much more rapid as less protein is extracted initially. Protein is dissolved by hot alkali but precipitated by cold trichloracetic acid.

The yield by either method was of the order of 1% of the tissue weight. Thus approximately 0·7 gm. of glycogen was obtained from each liver.

For some of the later experiments a large quantity of TCA glycogen was obtained from a horse liver by the above method.

Properties of glycogen.

The final analyses of the two glycogens were essentially identical

e.g. (1) Conversion to glucose on hydrolysis and estimation of reducing power was 98% - 100% for both

(2) $[\alpha]_D^{16}$ in H_2O OH-glycogen + 194°
TCA-glycogen + 190°

(3) Conversion into maltose by β-amylase
OH-glycogen 41%
TCA-glycogen 45%

RESULTS AND DISCUSSION.

The data for the various samples are shown in Table I.

Fig. 13 shows the dependence of sedimentation coefficient upon concentration for OH- and TCA- glycogens. These results were evaluated by the method of least squares to give an equation

$$S = S_o (1 - kc)$$

where k for OH-glycogen was 0·12 and for TCA-glycogen 0·11.

These results are in good agreement with those found in the previous section for the apparently monodisperse glycogens from several sources.

The concentration dependence of diffusion coefficient was investigated by Dr. W.A.J. Bryce. The results are shown in Fig. 13 Any dependence would appear to be negligible and within the error of the experimental technique.

The molecular weights in Table I indicate a considerable difference between OH- and TCA- glycogens. In order to confirm this, sedimentation coefficient distributions were calculated for one sample of OH-glycogen and two of TCA-glycogen. These distributions were corrected for diffusion but not for concentration;

Concentration dependence of S_{20} and D_{20}

(1) OH- glycogen-1

(2) TCA-glycogen 1

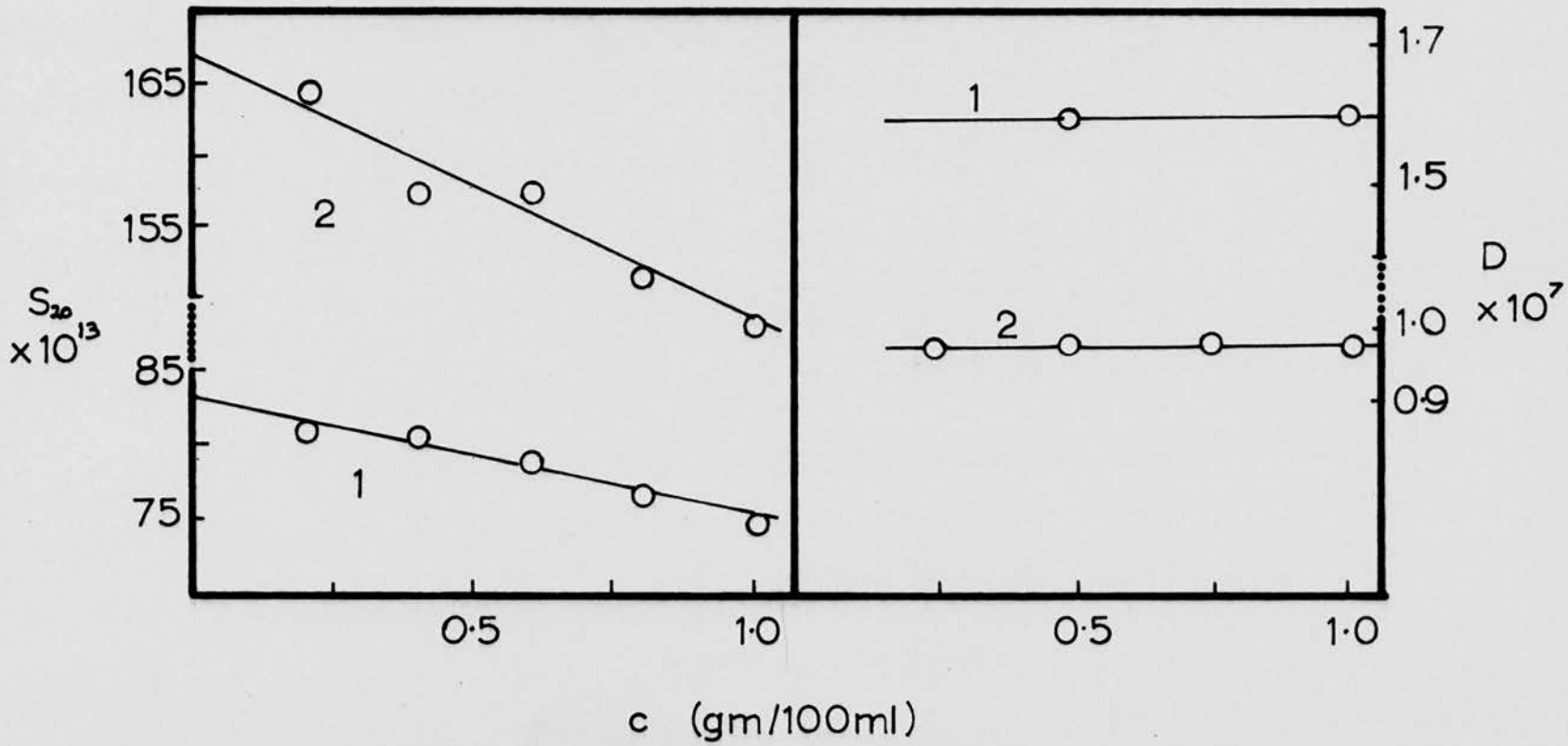


FIG. 13

FIG. 14

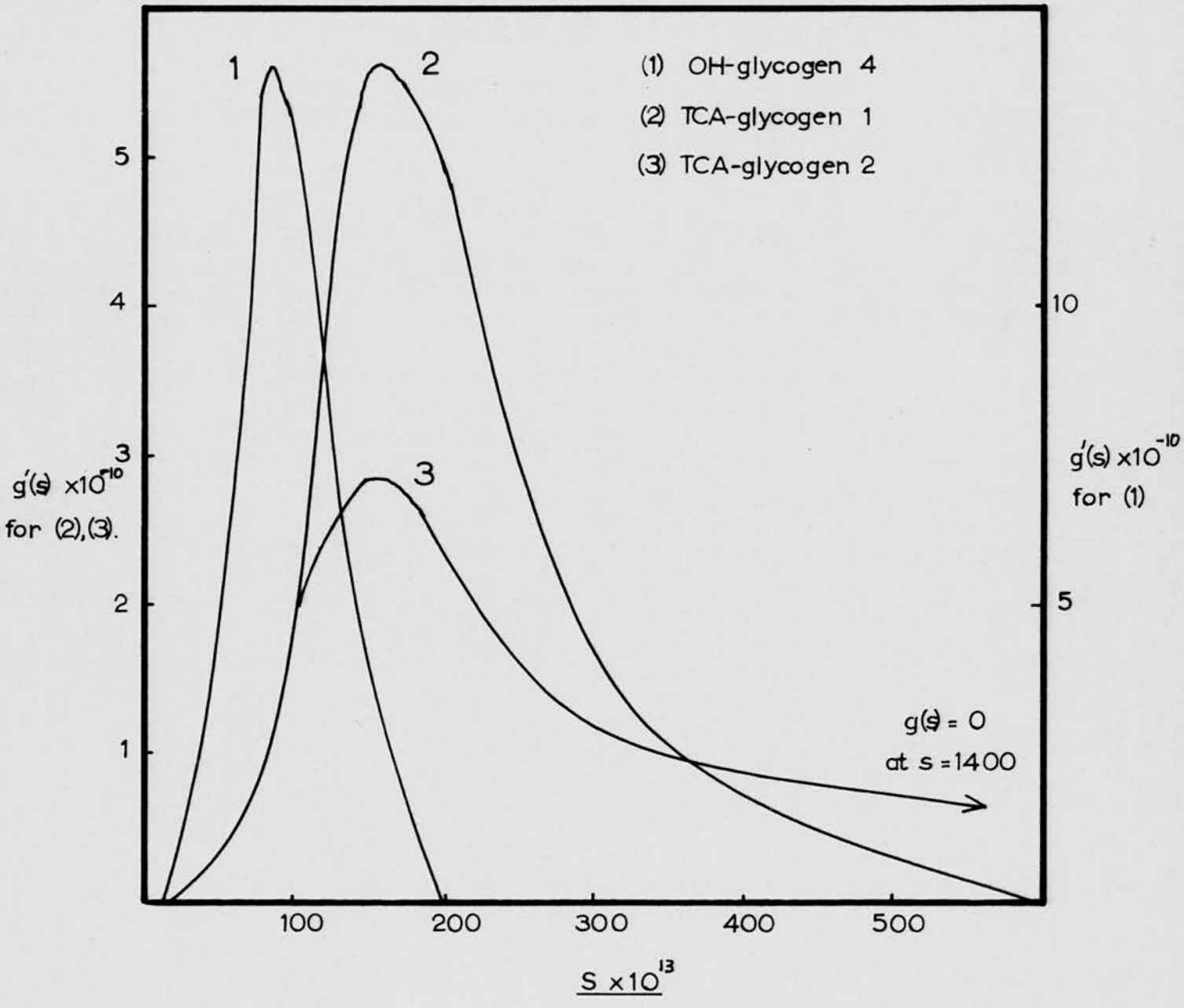

(1) OH-glycogen 4
(2) TCA-glycogen 1
(3) TCA-glycogen 2
1
2
3
g(s) = 0
at s = 1400
g'(s) x10^-10 for (2),(3).
g'(s) x10^-10 for (1)
S x10^13
100
200
300
400
500
1
2
3
4
5
5
10

TABLE I.

		$10^{13}(S_{20})_o$	$10^{-6}M_{SD}$	$10^{-6}\overline{M}_w$	I_{45}/I_{135}	$\overline{M}_w/M_{SD}$
OH-Glycogen	1	84	3·4	19·0	1·70	5·6
	2	86	3·6	-	-	-
	3	94	4.2	7.8	1·20	1·85
	4	95	4.2	8·0	1,20	1·9
TCA-Glycogen	1	168	10·8	(62·0 (63	2·00) 1.94)	5·7
	2	173	11·3	(160 (162	2·40) 2·40)	14·3
	3	163	10·3	-	-	-
TCA-Horse liver Glycogen		160	10·0	60	1·73	6·0

TABLE II.

		$10^{13}\ S_{20}$	$10^{13}\ \overline{S}_w$	$10^{13}\sigma$	Sk
OH-glycogen	4	87	95	34	+ 0·23
TCA-glycogen	1	150	220	99	0·70
	2	150	417	310	0·87

the validity of this correction for such wide distributions as those of TCA glycogen would be doubtful. All measurements were made at the same concentration and so the distributions ought to be comparable. These are shown in Fig. 14 The amount of overlap is small as only 8% of OH-glycogen has $S > 150$. while the corresponding proportions for the other two samples are 61% and 72%.

The mean sedimentation coefficients were calculated from these curves by

$$\bar{S} = \Sigma [g(S).\, S] / \Sigma\, g(S)$$

This is a weight average sedimentation coefficiemt.

The standard deviation (σ) was calculated from

$$\sigma = \sqrt{\mu_2 - (\mu_1)^2}$$

where μ_1, μ_2 are the first and second moments about the mean of the curve.

The final quantity to be calculated was the "Skewness". This is defined as the ratio of the separation between the mean and mode of the curve, to the standard deviation,

$$\text{i.e.} \quad (Sk) = |\text{mean} - \text{mode}| \,/\, \sigma$$

and is a measure of the departure of the distribution from symmetry.

These quantities are collected in Table II and emphasize the considerable difference between OH- and TCA- glycogen.

It is interesting to consider what type of molecular weight can be calculated from the mean sedimentation coefficient. Thus, if it is possible to obtain a weight average for both S and D, the

molecular weight calculated from these using the Svedberg equation will be

$$M_{ww} = \frac{RT}{1-\bar{V}\rho} \cdot \frac{\Sigma c_i S_i / \Sigma c_i}{\Sigma c_i D_i / \Sigma c_i}$$

On p. 50 , it was shown that

$$S_i = M_i (1 - \bar{V}\rho) / f_i$$

and $$D_i = RT / f_i$$

where f_i is the frictional coefficient. Also $c_i = n_i M_i / N$

Hence $$M_{ww} = \Sigma (n_i M_i^2 / f_i) / \Sigma (n_i M_i / f_i)$$

The average molecular weight resulting from the combination of weight average S and D cannot be a unique average, as it is dependent upon the frictional coefficient and must therefore vary with the molecular shape. Singer (1946) has shown that it is only for the "Free draining" coil model that this average assumes a simple form. For such a model, $f_i \propto M_i$ and hence

$$M_{ww} = \bar{M}_N$$

For other models, the average is more complex and, in particular, for spherical molecules where $f_i \propto M^{1/3}$

$$M_{ww} = \Sigma n_i M_i^{5/3} / \Sigma n_i M_i^{2/3}$$

Singer has shown that this average lies somewhere between a weight and a number average. For relatively narrow distributions, this effect is unimportant, but for the very wide distributions found for TCA-glycogens the divergence from a true weight average is serious.

To illustrate this, the distribution for TCA-glycogen (2)

was taken as a starting point and, assuming that a spherical model was applicable, corresponding distributions of D and M were calculated from the relations $D \propto S^{-1/2}$, $M \propto S^{3/2}$.

For arithmetical convenience it was assumed that

$$M = 5 \times 10^{6}, \quad D = 10^{-7} \text{ when } S = 100 \times 10^{-13}$$

Weight average S, D and M were calculated as

$$\overline{S}_w = 410 \times 10^{-13}$$

$$\overline{D}_w = 0{\cdot}636 \times 10^{-7}$$

$$\overline{M}_w = 47 \times 10^{6}$$

whence $$M_{ww} = 32 \times 10^{6}$$

Now $\overline{M}_w$ is the molecular weight which would result from light scattering and hence, even when a weight average S can be calculated good agreement between M_{ww} and light scattering results cannot be expected. Further, as the labour involved in calculating $\overline{S}_w$ for each sample would be prohibitive, it is usual to employ the modal sedimentation coefficient in conjunction with a weight average diffusion coefficient to calculate a molecular weight. In the above case, this molecular weight would be

$$M_{SD} \propto S \text{ mod.} / \overline{D}_w = 11{\cdot}8 \times 10^{6}$$

which is vastly different from the weight average. In fact, this value is not strictly an average value for the system, but corresponds fairly well to the molecular weight of the most abundant component, which is in this case approximately 10^{7}.

It would therefore appear that a unique molecular weight average cannot be calculated from sedimentation and diffusion

coefficients. However, as the molecular weight calculated from the modal sedimentation coefficient corresponds with reasonable accuracy to the molecular weight of the component with that sedimentation coefficient, it is possible to establish a relation between sedimentation coefficient and molecular weight even when using samples of wide distribution.

For four samples ranging from $S = 86 \times 10^{-13}$ to $S = 209 \times 10^{-13}$ a relationship was found between molecular weight and sedimentation coefficient as

$$M = 2{\cdot}70 \times 10^{3}\ S^{1{\cdot}59} \qquad (S \text{ in Svedbergs}).$$

A useful qualitative measure of polymolecularity is the ratio $\bar{M}_w / M_{SD}$ which - although having no theoretical significance - indicates very satisfactorily any difference in distribution. This is well illustrated by TCA glycogens (1) and (2), where the very large difference suggested by the ratios of $\bar{M}_w / M_{SD}$ is borne out by the measured distributions (Fig. 14).

In conclusion, it is obvious that the range of apparent molecular size present in TCA-glycogens is very much greater than in OH-glycogens and therefore the acid extraction method would appear to be the more satisfactory. However, there is the possibility that the extremely large molecular weight of TCA-glycogen may be attributable to the presence of aggregated material. This point will be considered in the next section.

III C.

Aggregation in TCA-glycogen.

For any polymer of large molecular weight, it is important to establish whether a true molecular dispersion or aggregates are present in solution. This is particularly the case for TCA-glycogen where the molecular weight is so very large. Aggregation may be due either to physical molecular entanglement, or to chemical hydrogen bonding or complexing through protein impurity (the amount of protein required could be well below the limit detectable by analytical means).

The absence of any abnormal behaviour of TCA-glycogen at low concentration in both light scattering and ultracentrifugation, suggests that purely physical aggregation is unlikely. It would be expected that disaggregation might occur at low concentration, but no evidence for this was found. Further, ultrasonic irradiation experiments made by Dr. W.A.J. Bryce failed to bring about any degradation under conditions which cause rapid degradation in amylopectin.

Preliminary experiments with TCA-glycogen-(1) showed that a decrease in molecular size occurred when a 0·5% solution in water was heated at 100° in air. The decrease was observed by ultracentrifugation and the results are given below.

Time (hours)	0	1	4	7
S_{20}	158	151	131	125

The overall degradation, as measured by sedimentation coefficient, is quite low and would appear to be practically complete after 4 hours. However, considerable changes took place

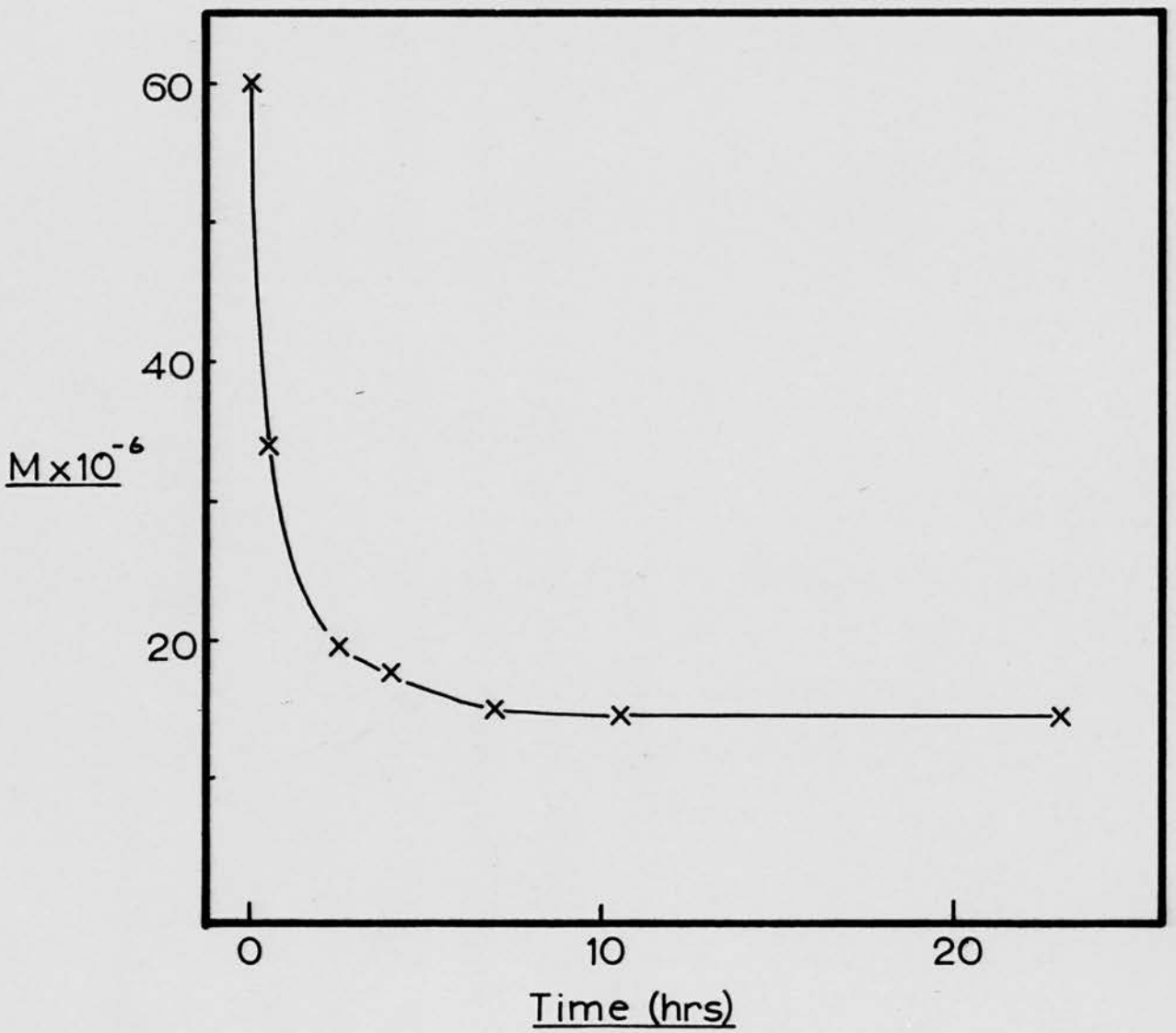
in the high molecular weight species present in the leading edge of the sedimentation patterns and, therefore, the reaction was followed by light scattering. A 0·2% solution of horse liver glycogen was boiled under reflux while passing nitrogen. Samples were removed at intervals and their molecular weights determined by light scattering. Fig. 15 shows that, after the initial very rapid decrease in molecular weight, little change occurs after 7 hours. The sample isolated after 20 hours was examined in the ultracentrifuge. $(S_{20})_o$ was 125×10^{-13} ($M_{SD} = 6{\cdot}5 \times 10^6$). Thus it would appear that disaggregation or degradation does occur on boiling TCA-glycogen solutions under oxygen-free conditions. This change must be mainly confined to the large molecular weight material since the ratio $\bar{M}_w / M_{SD}$ falls from 6·0 to 2·5, indicating considerable narrowing of the molecular weight distribution.

Pollard (1958) has suggested that glycogen should become less stable as the molecular weight increases because of the increased strain placed upon the bonds near the centre of the "tree-structure". In large glycogen molecules, these central bonds would tend to break under thermal agitation. This idea provides a possible explanation of the observed decrease in molecular weight on heating. However, similar degradation would occur if the large glycogen molecules were groups of smaller molecules bound together by some other weaker type of bonding - possibly through protein.

An experiment was made to distinguish between these two possibilities. A β -amylase limit dextrin was formed from TCA-glycogen. The molecular weight fell in proportion to the

FIG. 15

Degradation of HLG



amount of maltose released during enzyme action. This indicates that no internal bonds were broken and hence any protein-glycogen, or other weak bonds, were still intact. This dextrin was then heated at boiling point under nitrogen for 25 hours. A sample of the original glycogen was also heated for this time and from the product a β-amylase limit dextrin was prepared. The molecular weights of the various products are shown.

	S_{20}	M_{SD}	$\bar{M}_w$	$\bar{M}_w/M_{SD}$
(0) Horse liver glycogen (HLG)	160	10·0	60·0	6·0
(1) HLG - dextrin	100	4·5	36·0	8·0
(2) HLG-dextrin - boiled 25 hrs.	102	4·5	29·0	6·5
(3) HLG - boiled 25 hours	125	6·5	16·0	2·5
(4) HLG - boiled 25 hrs. -dextrin	85	3·4	7·9	2·3

If aggregation or specific weak bonds were present, samples (2) and (4) should be identical, as both were degraded to the same extent (44%) by β-amylase. However, these are obviously very different, particularly in the spread of molecular weight as indicated by $\bar{M}_w$ / M_{SD}. Thus much high molecular weight glycogen is still present in (2), in fact more even than in (3). These results suggest that heating does not cause the breaking of specific weak bonds, but rather that the stability of a glycogen molecule is directly related to its size.

As TCA-glycogen is not aggregated, considerable degradation must occur in the alkaline method of extraction. From the foregoing results, heating alone would be expected to cause

extensive degradation, but this cannot account completely for the difference as the molecular weight after boiling for 25 hours is still larger than that of OH-glycogen. To demonstrate that the difference is due to degradation and not to a different efficiency of extraction, TCA-glycogen was treated with 30% sodium hydroxide at 100° and products isolated at intervals by direct precipitation with ethanol. Light scattering measurements could not be made on these materials as, after this treatment, the products were discoloured and difficult to clarify (cf. Stetten et al, 1957). The sedimentation coefficients at 0·8% concentration were measured and are given below.

Time (hours)	0	¼	½	1	4
S_{20}	168	102	100	81	84

Very rapid degradation has occurred, but a limit is apparently reached within one hour. This accounts for the failure to find any degradation of OH-glycogen by strong alkali reported by Staudinger (1948). This limit can be explained by current ideas of alkaline degradation of polysaccharides (Whistler and BeMiller, 1958). According to these theories, degradation of a branched polysaccharide from the reducing end group eliminates side branches as resistant molecules with a saccharinic acid end group. In glycogen, a series of "alkali-dextrins" of molecular weights ½, ¼, ⅛ etc. of the original would be produced. It is easily shown that the weight average molecular weight of such a mixture would be approximately one third of the original. This agrees

quite well with the observed values of approximately 15 x 10^6 for heated TCA-glycogen and 4 - 8 x 10^6 for most of the alkali extracted glycogens on pp. 85 and 93 .

In conclusion, it can be said that glycogen extracted by the trichloracetic acid method is more representative of "native" glycogen than is alkaline extracted glycogen, which is degraded both thermally and specifically by the alkali.

III D.

Subfractionation of Glycogen.

For investigations of hydrodynamic behaviour, it is desirable to have material of relatively narrow molecular weig ht distribution to minimise the errors introduced by the different average values of molecular weight and dimensions which are measured. Various methods of subfractionation were considered.

(1) Fractional precipitation.

A 0·1% solution of TCA-glycogen was held in a constant temperature bath at 25°. Ethanol was added gradually to the solution with constant stirring until a faint permanent turbidity was produced. The solution was then heated to 35°, when the turbidity disappeared. On cooling slowly to 25° with stirring, the precipitate which reformed was collected by centrifugation after standing at 25° overnight. Further additions of ethanol were then made to give, in all, five fractions. However, on examination of these fractions in the ultracentrifuge, all were found to be identical; there was no difference in either S_{20} or the apparent width of the pattern.

Similar results were obtained when methanol and glycerol were used as precipitants. Stetten, Katzen and Stetten (1956) have, however, reported the fractionation of TCA-glycogen by cooling a 0 1% solution containing 15% ethanol to 0° and collecting fractions by centrifuging at intervals. This method was used on TCA-glycogen (1) and three fractions were obtained as shown.

	Yield(%)	$(S_{20})_o$	$M_{SD} \times 10^{-6}$	$M_w \times 10^{-6}$	$\bar{M}_w/M_{SD}$
Subfractions	45	170	10·8	66	6·1
	30	140	7·7	55	7·1
	20	160	9·4	50	5·3

Fractionation was negligible and, in particular, the ratio of $\bar{M}_w$ / M_{SD} varied but little. Further, qualitative examination of the sedimentation diagrams indicated no change in the spread of the boundary.

(2) Fractional solution.

Fractionation of dextran by solution of the polymer, suspended on "Celite", in a continuously improving solvent, has been shown to yield sharp fractions (Granath, 1958). This method was tried on horse liver glycogen.

200 ml. of a 1% solution of glycogen was stirred vigorously with 30 gm. "Celite" while the glycogen was precipitated by the addition of 2 volumes of ethanol. The "Celite", now coated with glycogen, was poured as a slurry in ethanol into a 3 cm. diameter jacketed column. Ten mixtures of ethanol and water were prepared ranging from 40% to 20% ethanol. 100 ml. of each was passed through the column at 22·5° and the effluent collected. Glycogen was precipitated by the addition of excess ethanol. These fractions were again found to give identical sedimentation patterns. Similar results were obtained when using cellulose powder and 0·1 mm. glass beads as a support.

The above methods based upon differential solubility are

therefore not satisfactory for fractionation of high molecular weight glycogen. This probably indicates that, for molecular weights in this range, the solubility of such a highly branched polymer varies only slightly, if at all, with molecular weight.

(3) Differential centrifugation.

As solubility does not differentiate between molecules of different weight, it is necessary to make direct use of the difference in molecular weight. If a solution of glycogen is ultracentrifuged, it is obvious that the largest molecules will be completely precipitated most quickly. Hence, by centrifuging a solution at different speeds or for different times, some degree of fractionation must be achieved (Stetten et al, 1956). This method was used to fractionate TCA-glycogen (2) into three fractions.

A 1% solution was centrifuged at 12,000 r/m for 30 minutes. The supernatant was decanted and respun at 17,000 r/m for 1 hour. The precipitated gel was in each case re-dissolved and the glycogen precipitated with ethanol. A third fraction was obtained from the second supernatant by precipitation with ethanol. The fractions were as shown.

Yield %	S_{20}	M_{SD}	$\bar{M}_w$	$\bar{M}_w / M_{SD}$
38	-	-	250	-
42	209	13·7	90	6·6
16	150	8·6	13·3	1·5

For the first fraction, it was not possible to measure sedimentation coefficients because of the extreme width of the sedimentation boundary. However, it was obvious that fractionation

had occurred and therefore a more careful fractionation scheme was devised and carried out on a horse liver glycogen.

100 ml. of a 2% solution was centrifuged for 1 hour at each of the following speeds - 11,800 r/m, 16,200 r/m, 25,980 r/m and 42,040 r/m. Fractions (1) - (4) were obtained from the sediments and fraction (5) from the final supernatant. Fractions (1) - (4) were re-dissolved and each divided into subfractions (a) and (b) by centrifugung again for 30 minutes at the same speed. Each of the subfractions 1(a) - 3(b) was again divided into two, a(i), a(ii), etc., by spinning again at the same speed. The fifteen fractions were examined in the ultracentrifuge. The modal sedimentation coefficient and also the minimum and maximum coefficients were measured. The results are shown in Table I.

These results again show the remarkably wide distribution of molecular weight present in a trichloracetic acid extracted glycogen. However, although fractionation has been achieved, it is evident from the minimum and maximum values of sedimentation coefficients that, within each fraction, a considerable spread of molecular size exists and that, in fact, in no case has a sharp fraction been produced.

Thus none of the methods used has given good fractionation, and it was felt that no advantage would be conferred by using these fractions for hydrodynamic measurements.

TABLE I.

Fraction	Yield %	$S_{20} \times 10^{13}$ Min.	Mode	Max.
1a(i)	2·5	600	1100	2500
1a(ii)	1·5	300	800	1600
1b(i)	7·0	0	700	1500
1b(ii)	4·0	0	170	800
2a(i)	3·5	220	660	1400
2a(ii)	2·5	0	500	1200
2b(i)	8·0	0	400	900
2b(ii)	5·0	0	190	600
3a(i)	8·0	100	340	800
3a(ii)	5·0	0	160	520
3b(i)	14·0	80	170	460
3b(ii)	11·0	0	146	500
4a	13·0	0	140	370
4b	9·0	40	100	240
5	5·0	0	76	180

III E. MOLECULAR SHAPE OF GLYCOGEN IN SOLUTION.

Introduction.

Information about the shape of the glycogen molecule in solution has been obtained from measurements of viscosity, sedimentation and light scattering. The relevant data for three samples are collected in Table I.

Sample	$[\eta]$	$(S_{20})_o \times 10^{13}$	$M_{SD} \times 10^{-6}$	M_w	ν
OH glycogen I (OH-G)	6·2	84	3·4	(19·0 4·0	1·7) * 1·18
TCA glycogen I(TCA-G)	7·1	168	10·8	62	1·94
(TCA) Horse Liver glycogen (HL-G)	7·8	160	10·0	60	1·73

* This sample was polydisperse and was therefore partially fractionated by centrifuging at 20,000 r/m for 15 minutes.

The partial specific volume, $\bar{V}$, was taken as 0·62 ml/gm. for all samples. This volume is not necessarily the actual hydrodynamic volume of the glycogen in solution as hydration of the molecules may occur. For interpretation of viscosity and sedimentation measurements, the actual hydrodynamic volume V_h is required. Two different approaches to the determination of V_h were made. The simpler and more direct involved the determination of the sedimentation volume of glycogen by centrifuging a solution at high speed to form a gel. The alternative method was to measure the sedimentation rate of glycogen in solutions of different density. Extrapolation was then made to that density at which the sedimentation rate was zero, when the density of the molecules must equal the solution density.

Experimental.

I. A stainless steel centrifuge tube was used as a pyknometer and its volume determined using nitrobenzene. The tube was filled with nitrobenzene and the contents allowed to come to temperature equilibrium in a bath at 22·5°. A cover glass was carefully slid over the mouth of the tube which was then removed from the bath, dried and weighed. Approximately 0·180 gm. of horse liver glycogen was added to the cleaned tube, dissolved in about 8 ml. water and centrifuged for three hours at 42,000 r/m in the preparative rotor of the Spinco Ultracentrifuge. Most of the glycogen had then sedimented as a gel. The glycogen remaining in solution was estimated by hydrolysis to glucose and determination of reducing power. The tube was thoroughly drained and excess solution carefully removed with filter paper before weighing. The volume occupied by the sediment was determined by filling with nitrobenzene as before and re-weighing. Hence, knowing the volume of sediment and weight of glycogen present, the sedimentation volume could be calculated as 2·68 ml/gm.

II. In order to make reasonably accurate extrapolation to the density of zero sedimentation rate, a wide range of density (1·0 - 1·35) must be covered. This can be achieved by the addition of low molecular weight solutes to the aqueous solution of glycogen. However, the viscosity of the solution is also increased in this way and of the three solutes, potassium iodide, sucrose and chloral hydrate, which were tried, only the last was found to give manageable solutions.

A series of six solutions of the same concentration of glycogen (Commercial II) and differing concentrations of chloral hydrate were prepared. Their densities were determined using a density gradient tube calibrated with potassium iodide solutions (Section III E). The sedimentation coefficients were determined and the relative viscosities measured at 20°. To correct for the increased viscosity of the solution, the observed sedimentation coefficient was multiplied by the relative viscosity. These corrected values were then plotted against density of solution and extrapolated to zero sedimentation coefficient. (Table II and Fig. 16)

Density	η_r	S_{20}	$S_{20} \cdot \eta_r$
1·00	1·00	76·5	76·5
1·080	1·46	50·3	73·5
1·155	2·12	32·5	69·0
1·226	3·13	18·5	58·0
1·290	4·67	11·0	51·5
1·350	7·10	5·8	41·2

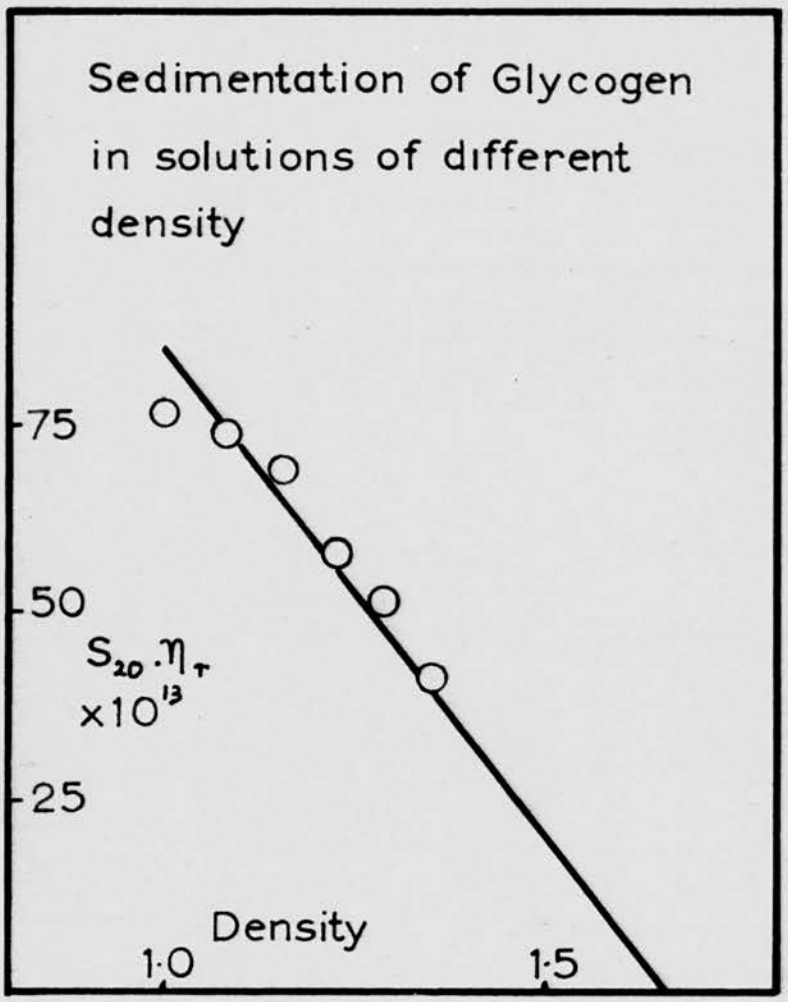
Extrapolated value = 1·65 gm/ml.

Hence, volume in solution = 0·60 ml/gm.

Discussion.

The sedimentation volume cannot be interpreted directly as the volume of the glycogen molecules in solution. Even if the moledules were perfect uniform spheres and packed in the closest possible way, they would occupy only some 74% of the sedimentation

FIG. 16


Sedimentation of Glycogen
in solutions of different
density
75
50
25
$S_{20} \cdot \eta_T$
$\times 10^{13}$
Density
1·0
1·5

volume. The maximum value for the hydrodynamic volume must then be 74% of 2·68 ml/gm., i.e. 1·98 ml/gm. However, the theoretical maximum of 74% appears to be attained very seldom in practice. Even for glass spheres in a non-interacting medium, the highest value found was 64% (Bloomquist and Shutt, 1940). Where interaction at the surfaces can occur, very much lower results - 6% - have been obtained (Vold, 1959). Further, Chomse (1951) has shown by model experiments that non-spherical particles always give looser packing. For a material such as glycogen, in aqueous solution, interparticle forces are likely to be important and therefore, both on this count and on that of asymmetry, the volume fraction of glycogen in the sediment must be less than the theoretical 0·74. The value of 1·98 ml/gm. must be therefore an overestimate of the hydrodynamic volume.

The second method suggests that the hydrodynamic volume is the same as the partial specific volume. However, this procedure is not free from criticism. Thus although it shows that in a solution of density 1·65 gm/ml. the glycogen molecules would also have that density, it does not establish beyond doubt that in water the molecular density would be the same. High concentration of chloral hydrate might tend to dehydrate the molecules. Alternatively, if solvent were not specifically bound to the glycogen, but merely immobilised within the coils of the molecules, the density would change with the density of the bulk of the solution and, hence, the density obtained by extrapolation would be the unhydrated value.

It is not, therefore, possible to establish unambiguously the hydrodynamic volume. However, it is of interest to calculate the molecular shape corresponding to the two extreme values, in the knowledge that the actual shape must be somewhere between these extremes.

On p. 97, it was shown that a relationship between molecular weight and sedimentation coefficient of the form $M \propto S^{1.59}$ was obeyed. From Stokes' Law it can be shown that for rigid spheroidal molecules, $M \propto S^{1.5}$. This agreement is probably within experimental error and, therefore, it seems more satisfactory to treat the hydrodynamic behaviour of glycogen on the basis of a rigid spheroid rather than as a coiled molecule, which would require that $M \propto S^{2.0}$ (Kuhn and Kuhn, 1943).

The viscosity was interpreted in terms of the theory of Simha (1940). It is not possible to use the equation quoted on p. 45 as this is valid only for $J \gg 1$. For low values, the function has been computed by Mehl, Oncley and Simha (1940) and their tabulated values were used here. These results were also compared with those calculated from the more simple equation of Kuhn (1933),

$$\text{viz.} \qquad \eta_{sh}/\phi = 2.5 + J^2/16 \qquad (1)$$

where ϕ - the volume fraction of solute - is equal to CV_h (C in gm/ml.) and hence $\eta_{sh}/\phi = [\eta]/V_h$

Frictional ratios were calculated from sedimentation data as detailed on p. 51. The method of Kraemer (1940) (p. 51) was used to correct for the increased molecular volume resulting from

hydration. Axial ratios were then calculated using Perrin's equation (p. 51).

These results are collected in Table II.

	V_h = 1·98 ml/gm			V_h = 0·62 ml/gm.		
	OH-G	TCA-G	HL-G	OH-G	TCA-G	HL-G
$[\eta]/V_h$	3·1	3·6	3·9	10·0	11·4	12·6
J (Simha)	2·4	2·9	3·3	8·0	9·0	9·5
J (Kuhn)	3·1	4·2	4·7	10·9	11·9	12·7
f / f_o	1·0	1·10	1·10	1·45	1·57	1·57
J (Perrin)	1·0	2·8	2·8	8·5	10·5	10·5

For the unhydrated case the values agree reasonably well. The simple equation of Kuhn gives values not greatly different from those derived by the more soundly founded theory of Simha. For the higher limit of hydrodynamic volume, the axial ratios for the two larger molecular weight samples are in good agreement. These methods, based independently upon either viscosity or sedimentation measurements, thus give values of J ranging from near unity to 10 with the higher values almost certainly being a better estimate.

It would obviously be more satisfactory to attempt to determine the molecular shape without making any assumption of particle volume. One method of achieving this is to use the treatment of Scheraga and Mandelkern (1953) which combines the results of sedimentation and viscosity measurements in an equation

$$\beta = N S [\eta]^{1/3} \eta_0 / M^{2/3} (1 - \bar{\nu}\rho) \qquad (2)$$

where β is tabulated in terms of axial ratio. For the

three samples, β was found as 2·33, 2·24, 2·33 respectively. These correspond to axial ratios of 7, 5 and 7. The magnitude of the difference represents experimental error and this method therefore suggests an axial ratio of the order of 6.

An alternative treatment by Ogston (1953) combines measurements of sedimentation coefficient, concentration dependence of sedimentation coefficient and intrinsic viscosity in an equation

$$\zeta/k' \cdot \frac{S_o \, (d(1/S)/dc)}{[\eta]} = F(J), \qquad (3)$$

where ζ is a constant = 0·667 and k' a constant whose value, although not certain, lies between 1·8 and 3·0.

For glycogen, where $S = S_o (1 - kc)$

$$d(\tfrac{1}{S})/dc = k/S_o \quad \text{at} \quad c = 0$$

Hence, $$F(J) = \zeta k/k'[\eta]$$

k = 0·11 for TCA-G and for HL-G and k = 0·12 for OH-G. Taking k' = 3·0, J = 6·3, 8·0, 8·8 respectively and for k' = 1·8 the values were 3·0, 4·2, 5·0. These again give an average axial ratio of 6.

This axial ratio can be used to estimate the hydrodynamic volume. An average value of 0·9 ml/gm. was obtained.

Thus hydrodynamic methods yield a value of the axial ratio of the order of 6 and suggest that the effective volume of glycogen in solution is slightly greater than in the dry state.

An estimate of molecular dimensions can also be made from light scattering measurements. Unless the molecular shape is known, however, it is only possible to determine the radius of gyration - ρ (p. 18). For a definite model, ρ can be evaluated in terms of a characteristic dimension. For ellipsoids of revolution, of major semi-axis "a" and axial ratio J, ρ is given by

$$\rho = \left(\frac{1 + 2/J^2}{5}\right) \times a \quad \text{(Guinier 1943)} \qquad (4)$$

For glycogen the radius of gyration is fairly small and can therefore be determined with sufficient accuracy from measurements of dissymmetry (z), since it is easily shown that

$$z \approx 0{\cdot}156\, \lambda'(z - 1)^{1/2} \qquad (5)$$

where λ' is the wavelength of the light in solution.

Hence for the mercury green line ($\lambda' = 410\,m\mu$)

$$\rho = 63{\cdot}7\ (z - 1)^{1/2}\ m\mu$$

These equations allow the dimensions of an ellipsoid of given axial ratio to be calculated from the dissymmetry of light scattering. Dimensions can also be calculated from the molecular weight for it is readily shown that

$$a = (4{\cdot}0\ \bar{V}\, M\, J^2 \times 10^2)^{1/3} \qquad (6)$$

and hence comparison of equations (4) and (6) should allow the determination of J.

For the three samples

	J(V = 0·62)	J(V = 1·9)	J(V = 0·90)
OH-G	14·3	8·0	11·8
TCA-G	13·1	7·4	10·8
HL-G	10·5	5·9	8·3

These results yield an average axial ratio of 10 for the most probable value of the hydrodynamic volume. This value is inevitably an overestimate as the radius of gyration obtained from light scattering is a z-average compared with the weight average molecular weight. Taking this fact into consideration, the results above accord reasonably well with those derived from hydrodynamic behaviour and it is therefore suggested that the shape of glycogen in solution is best represented by an ellipsoid of axial ratio approximately 6.

SECTION IV.

THE DEGRADATION OF GLYCOGEN BY β -AMYLASE.

INTRODUCTION.

The enzyme, β - amylase degrades starch-type polysaccharides with the production of maltose. Degradation occurs from the non-reducing terminal groups and the enzyme appears to be completely specific for 1-α-4 bonds. Thus its attack upon the branched polysaccharides, glycogen and amylopectin, is halted when the outer chains have been degraded to within two or three glucose units of the 1 - 6 branch points. The extent of degradation is 40 - 50% for glycogen and 50 - 70% for amylopectin. (Review by Whelan 1958).

Although the general course of the reaction is well-established, little work has been carried out to investigate the specific action-pattern. In general, two different types of action pattern are recognised for enzymically catalysed degradation reactions. The enzyme may either completely degrade one molecule at a time ("single-chain" action) or make a random attack upon all molecules ("multi-chain" action). While the latter might appear to be the obvious mechanism as the reaction is essentially stepwise, the former has the energetic advantage that the activation energy might be conserved throughout the degradation of one molecule.

Conflicting evidence as to the action-pattern of the degradation of amylose by β -amylase has been presented. The earliest results (Kerr, 1949) indicated a single-chain action but this was later opposed (Bourne and Whelan, 1950) and a multi-chain action suggested. Still more recently, Cowie et al (1958) have confirmed the original single-chain hypothesis.

No corresponding studies of β -amylase action on either glycogen or amylopectin have been reported. However, several studies (Larner et al 1956, Cori 1958) have been made on phosphorylase, an enzyme which degrades branched glucosans to a slightly smaller extent than β -amylase with the liberation of glucose - 1 - phosphate instead of maltose. The action-pattern of this enzyme on glycogen was investigated by following molecular weight distribution during degradation. The results of these workers are somewhat at variance. Larner (1956) showed that the high molecular weight material was preferentially degraded while the lower molecular weight material was largely unaffected. Thus very little shift in the maximum of the distribution curve was observed. Cori's results (1958) suggested that the enzyme had no molecular weight specificity, as the changes in the distribution curves during phosphorolysis showed all molecular weight species were degraded to the same extent. However, Cori does not clearly specify his experimental conditions and this divergence - if not merely an error in interpretation - is probably attributable to different conditions of reaction.

For a highly branched molecule, a multi-chain degradation seems most probable as the enzyme action must be halted by a branch point after removal of only a few maltose units. At this point, the enzyme / substrate complex might easily break down, the enzyme to recombine with another molecule. However, the nearest molecule is always that which has already been partially degraded, and recombination might occur, leading to what would be effectively a

single-chain action. This appears to be the case in the enzymatic synthesis of dextran where Bovey (1959) has shown that a single-chain mechanism must be operative.

The questions of single- or multi-chain action and molecular weight specificity in the β -amylolytic degradation of branched polysaccharides therefore require investigation. The method adopted for following the degradation was the determination of the change in distribution of molecular weight by ultracentrifugal analysis. Since the practical and arithmetic difficulties of this technique are strongly affected by the extent of the concentration dependence of the sedimentation coefficient, glycogen was selected in preference to amylopectin. The use of glycogen also allowed a direct comparison with Larner's work on phosphorylase action.

EXPERIMENTAL.

Preparation of Glycogen Sample.

The glycogen used was prepared by the alkaline Pfluger method from rabbit livers. As described in Section III B, this glycogen was polydisperse and contained both a large and a small component. For distribution studies, it is desirable to have a starting material of fairly narrow distribution and therefore subfractionation was attempted. A 1·0% solution in 0·1 $\bar{M}$ NaCl was centrifuged at 20,000 g. for 15 minutes in the preparative rotor of the Spinco ultracentrifuge. A pellet of gel material was obtained and was discarded. Examination by ultracentrifugation showed the large molecular weight component to have been removed. The solution was then cooled to 0° and cold ethanol added until a definite turbidity developed. This precipitate was collected by centrifuging at 0°. The small molecular weight component remained in solution. On ultracentrifugation of the precipitated glycogen, an almost symmetrical peak was obtained with neither high nor low molecular weight components. While this material did not have an ideally narrow distribution, it was sufficiently so for the purpose of this work.

Enzyme preparation.

β - Amylase was isolated from soya-beans by the method of Peat, Pirt and Whelan (1952). It was not possible to obtain the enzyme in a crystalline form although attempts were made to this end. However, the purity of the preparation was demonstrated by several control experiments.

(a) Maltase - No significant amount of maltase was present as no increase in reducing power could be detected on incubation with maltose.

(b) Z - enzyme was shown to be absent by experiments on fractions of amylose obtained by aqueous dispersion (Cowie and Greenwood,1957). In these experiments, reproducible conversion limits of 60 - 80% were found, thus indicating the absence of Z - enzyme.

(c) α - Amylase The results of the work to be described in this section show the absence of this enzyme. Had α - amylase been present, the molecular size of the dextrin would have been of a very much lower order. Also, the molecular size of the limit dextrins produced by action of the enzyme on amylose demonstrates the complete absence of contaminating α - amylase (Cowie et al, 1958).
The activity of the enzyme preparation in Hobson, Whelan and Peat's units (1950) was 20,000 units / mg.

Preparation of Dextrins.

Glycogen, at a concentration of 1 mg / ml, was incubated with 50 units / mg. of β - amylase in 0·2 $\bar{M}$ acetate buffer, pH 4·6 at 35°. Preliminary experiments indicated that limiting conversion was attained in about two hours. However, to ensure complete conversion the digest was left for 24 hours before isolating the limit dextrin. The enzyme was deactivated by heating briefly on a boiling water bath, and the polysaccharide immediately precipitated from solution by addition of two volumes of ethanol. The precipitate was

collected by centrifugation, washed several times with alcohol, and finally dried with ether. A dextrin at an intermediate degree of conversion was similarly isolated.

Determination of degree of conversion.

The initial glycogen concentration was determined by hydrolysis to glucose, followed by estimation of reducing power by the ceric sulphate / alkaline ferricyanide method of Lampitt, Fuller and Coton (1955). The maltose liberated during enzymolysis was estimated by the same method. The reagents were calibrated against "Analar" grade glucose and maltose, since the reaction is not strictly stoichiometric.

The conversions found were

Intermediate dextrin	13·2%
Limit dextrin	41%

Sedimentation measurements.

The three samples were centrifuged in 0·1 $\overline{M}$ NaCl at a concentration of 0·8 gm/100 ml. All experiments were made at a speed of 20,410 r/m. The first photographs of the boundary were taken after this had completely cleared the meniscus, and thereafter 5 photographs were taken at 6 minute intervals. In order to obtain the time representing the effective start of sedimentation, log x was plotted against time (x is the distance of the boundary from the axis of rotation). The time on this curve, corresponding to

the position of the meniscus, was taken as the initial time for all later calculations.

Calculation of Molecular Weight Distribution.

Molecular weight distribution for the three samples were calculated according to the method described in Section II F. To illustrate the method, the calculation for the limit dextrin will be given in part.

Table I shows the calculation of $g^*(S)$ vs S for the photograph taken at 1,120 sec. A similar calculation was performed for each of the other photographs at 1,450 sec., 1,720 sec. and 2,020 sec. In order to eliminate diffusion, $g^*(S)$ at intervals of 10 S was plotted against 1/xt and an extrapolation to 1/xt = 0 made for each S value. The extrapolated values of $g'(S)$ were plotted against S as in fig. 17 curve (3). Fig. 17 also shows two of the $g^*(S)$ curves from which $g'(S)$ was derived.

$g'(S)$ vs S was converted into dc/dx vs x by

$$dc/dx = g'(S) \cdot x_o^2 \cdot c_o / x^3 w^2 t$$

and $$x_i = x_o \cdot \exp.(S_i w^2 t)$$

where t is the mean time of the experiment,

and $c = 0{\cdot}8$ gm/100 ml. (See Fig. 18)

For the Johnston-Ogston corrections this curve was divided into 17 planes at x intervals of 0·02 cm. At each plane S_i and the total concentration $(c_\pi)_i$ were calculated. From these $(S_o)_i$ was obtained using

$$S = S_o(1 - 0{\cdot}1c)$$ See Table II.

FIG. 17

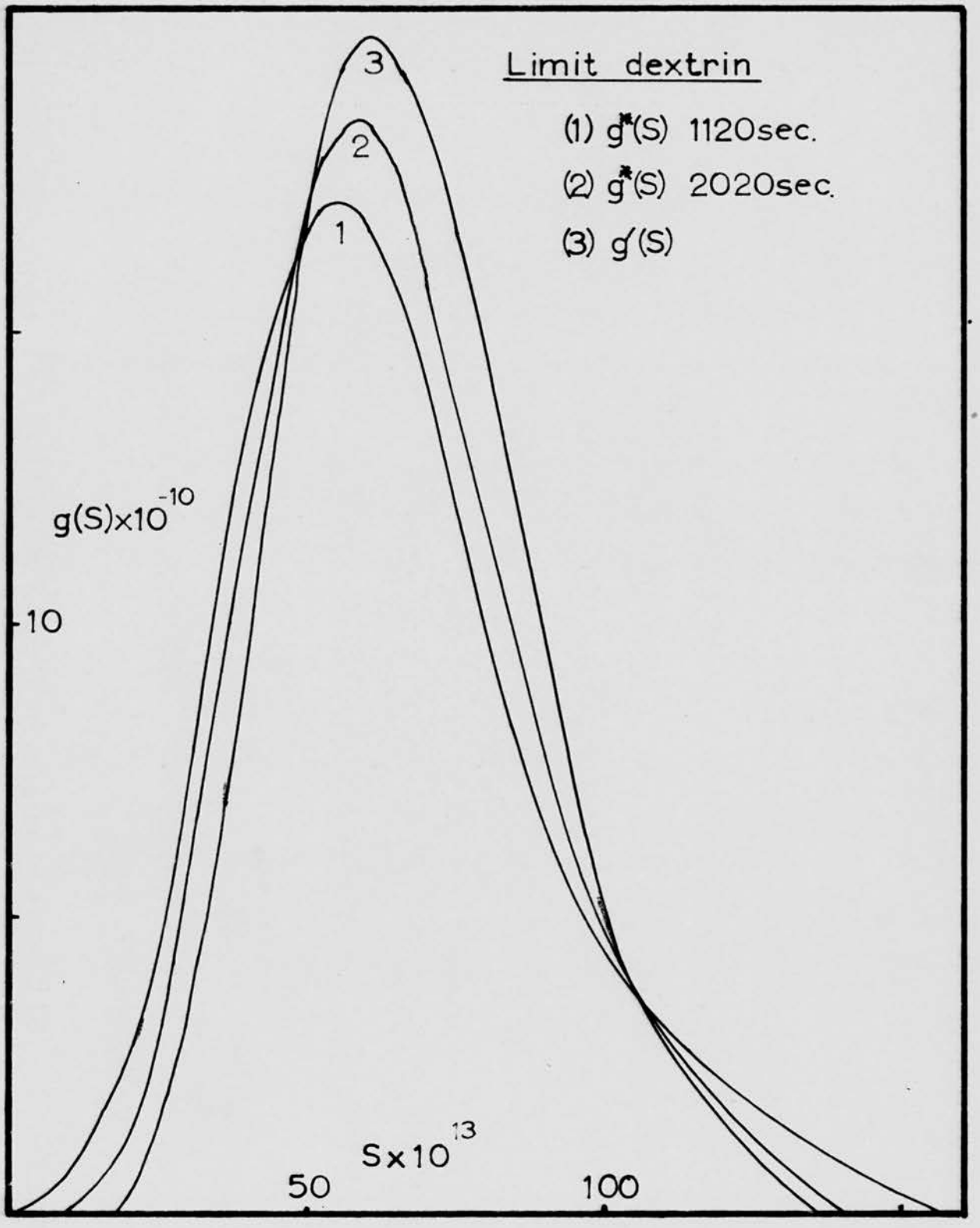

Limit dextrin
(1) g*(S) 1120sec.
(2) g*(S) 2020sec.
(3) g(S)
3
2
1
g(S)x10^-10
10
Sx10^13
50
100

FIG. 18

Limit Dextrin

$\frac{dc}{dx}$ vs x (corrected for diffusion)

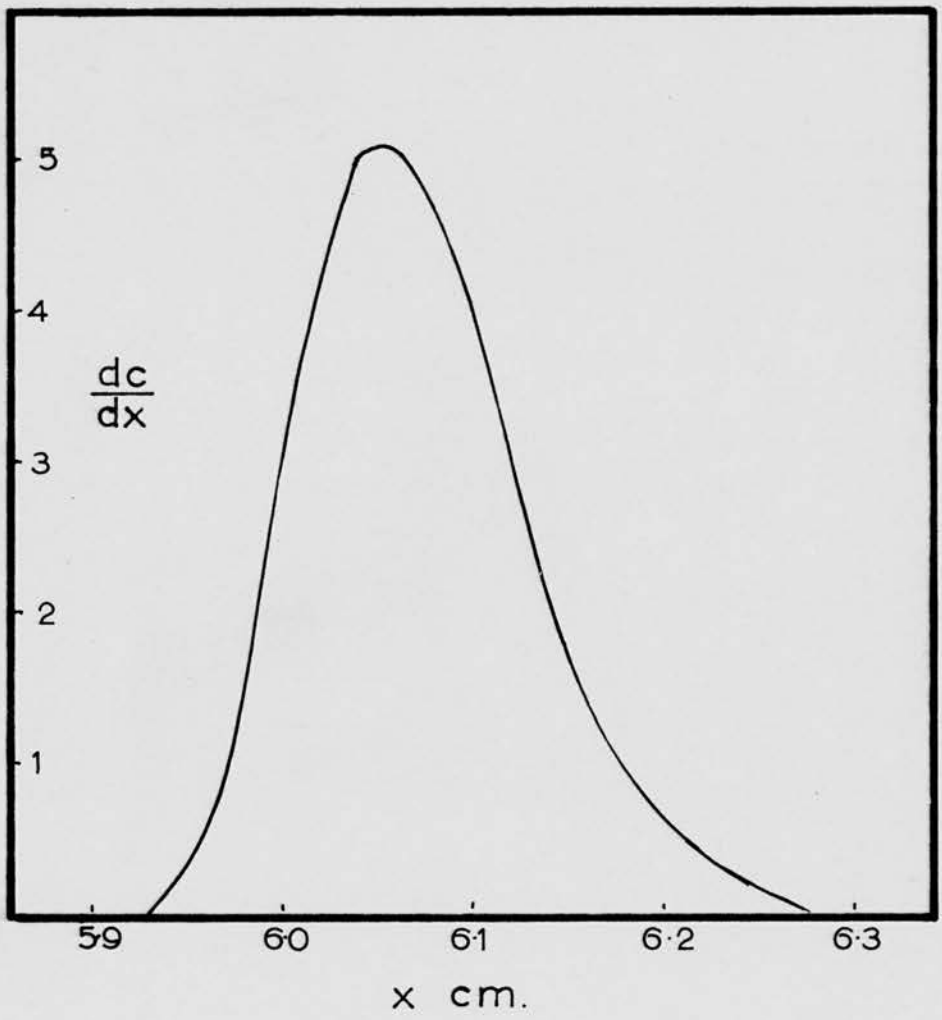


TABLE I

(2) Limit Dextrin.

x	(h)	x^2	hx^2	x/x_o	$\log_{10} x/x_o$	S	$g^*(S)$
5·888	0	34·67	0	1·0	0	0	0
5·913	1·0	34·95	34·95	1·00403	0·00175	7·88	0·36
5·938	4·0	35·26	141·04	1·00827	0·00358	16·13	1·61
5·963	11·0	35·56	391·16	1·01252	0·00541	24·37	4·54
5·988	23·0	35·85	824·55	1·01676	0·00722	32·53	9·78
6·013	33·5	36·16	1211·36	1·02101	0·00903	40·68	14·3
6·038	39·0	36·46	1421·94	1·02525	0·01083	48·79	16·9
6·063	39·0	36·76	1433·64	1·02950	0·01265	56·99	17·1
6·088	35·5	37·06	1315·63	1·03374	0·01441	64·92	15·8
6·113	28·5	37·37	1065·05	1·03799	0·01619	72·94	12·8
6·138	21·0	37·67	791·07	1·04223	0·01796	80·91	9·48
6·163	15·0	37·98	569·70	1·04648	0·01973	88·88	6·83
6·188	11·0	38·29	421·19	1·05072	0·02149	96·81	5·03
6·213	8·5	38·60	328·1	1·05497	0·02324	104·70	4·00
6·238	6·0	38·91	233·46	1·05921	0·02498	112·53	2·85
6·263	5·0	39·22	196·10	1·06346	0·02672	120·37	2·37
6·288	3·5	39·54	138·39	1·06770	0·02845	128·17	1·70
6·313	2·5	39·85	99·63	1·07195	0·03017	135·92	1·23
6·338	1·0	40·17	40·17	1·07619	0·03189	143·66	0·50
6·363	0·5	40·49	20·25	1·08044	0·03360	151·37	0·34
6·388	0	40·81	0	1·08468	0·03530	159·03	0·00

t = 1120 secs. $\omega^2 = 4{\cdot}565 \times 10^6$ $\dfrac{\Delta x \,.\, \Sigma hx^2}{x_o^2} = 7{\cdot}7$

TABLE II.

	x	dc/dx	$dc/dx\big]_{x=c_i}$	$c_T x$	S_i	$(S_o)_i$	C_i plateau
1	5·940	0·13	0·0026	0·0026	19·0	19·0	0·00252
2	5·960	0·53	0·0106	0·0132	26·3	26·33	0·00997
3	5·980	1·50	0·0300	0·0432	33·6	33·74	0·02734
4	6·000	2·98	0·0596	0·1028	40·8	41·22	0·05333
5	6·020	4·16	0·0832	0·1860	48·1	49·01	0·07444
6	6·040	4·98	0·0996	0·2856	55·2	56·8	0·09195
7	6·060	5.04	0·1008	0·3864	62·4	64·9	0·09666
8	6·080	4·65	0·0930	0·4794	69·6	73·1	0·09390
9	6·100	3·98	0·0796	0·5590	76·7	81·2	0·08460
10	6·120	3·05	0·0610	0·6200	83·8	89·3	0·06788
11	6·140	2·17	0·0434	0·6634	90·8	97·2	0·05014
12	6·160	1·35	0·0270	0·6904	97·9	105·1	0·03138
13	6·180	0·90	0·0180	0·7084	104·9	112·8	0·02058
14	6·200	0·61	0·0122	0·7206	112·0	120·6	0·01379
15	6·220	0·38	0·0076	0·7282	118·9	128·2	0·00859
16	6·240	0·27	0·0054	0·7336	125·9	135·8	0·00605
17	6·260	0·11	0·0022	0·7358	132·8	143·3	0·00244

Hence the sedimentation coefficient of component i changes at plane j by

$$(\Delta S_i)_j = -0{\cdot}1\,(S_o)_i\,\Delta c_{i-j}$$

At plane (1) component (1) only is present and

$$c_1 = 0{\cdot}0026\ ; \quad (S_1)_1 = 19{\cdot}0$$

At plane (2)

$$(\Delta c)_{2-1} = 0{\cdot}0106 \quad \therefore \quad (\Delta S_1)_2 = 0{\cdot}020$$

$$\therefore \quad (\Delta c_1)_2 = \frac{c_1\ (\Delta S_1)_2}{S_2 - (S_1)_2}$$

$$= \frac{0{\cdot}0026 \times (-0{\cdot}020)}{26{\cdot}3 - 19{\cdot}0 + 0{\cdot}02}$$

$$= 0{\cdot}00001$$

$$\therefore \quad (c_1)_2 = 0{\cdot}00259 \qquad c_2 = 0{\cdot}01061$$

$$(S_1)_2 = 18{\cdot}98$$

At plane (3) $\qquad (\Delta c)_{3-2} = 0{\cdot}0300$

$$(\Delta c_1)_3 = \frac{0{\cdot}00259\ (-0{\cdot}03 \times 1{\cdot}9)}{33{\cdot}6 - 18{\cdot}98 + 0{\cdot}057} = 0{\cdot}00001$$

$$(\Delta c_2)_3 = \frac{0{\cdot}01061\ (-0{\cdot}03 \times 2{\cdot}63)}{33{\cdot}6 - 26{\cdot}3 + 0{\cdot}079} = 0{\cdot}0001$$

$$\therefore \quad (c_1)_3 = 0{\cdot}00258 \qquad (c_2)_3 = 0{\cdot}01051 \qquad c_3 = 0{\cdot}03011$$

$$(S_1)_3 = 18{\cdot}92 \qquad (S_2)_3 = 26{\cdot}22$$

This calculation was carried out for each component present in all 17 planes. The corrected values of the concentrations at the 17th plane are the concentrations in the "plateau" region. These are shown in Table II.

In the correction for boundary sharpening the values of dS/dS_o were obtained by numerical differentiation of the S, S_o values in Table II.

Fig. 19 shows the $g(S_o)$ vs S_o curves for the three samples.

A distribution of molecular weight was calculated from each of the above curves using

$$M = 2{\cdot}70 \times 10^{3} \times S_o^{1{\cdot}59}$$

$$\text{and} \quad dS/dM = 2{\cdot}31 \times 10^{-17} \times S_o^{-0{\cdot}591}$$

S_o values were converted directly to M values and $g(S_o)$ into $g(M)$ by

$$g(M) = g(S_o)\, dS/dM$$

The resulting distributions are shown in Fig. 20.

FIG. 19

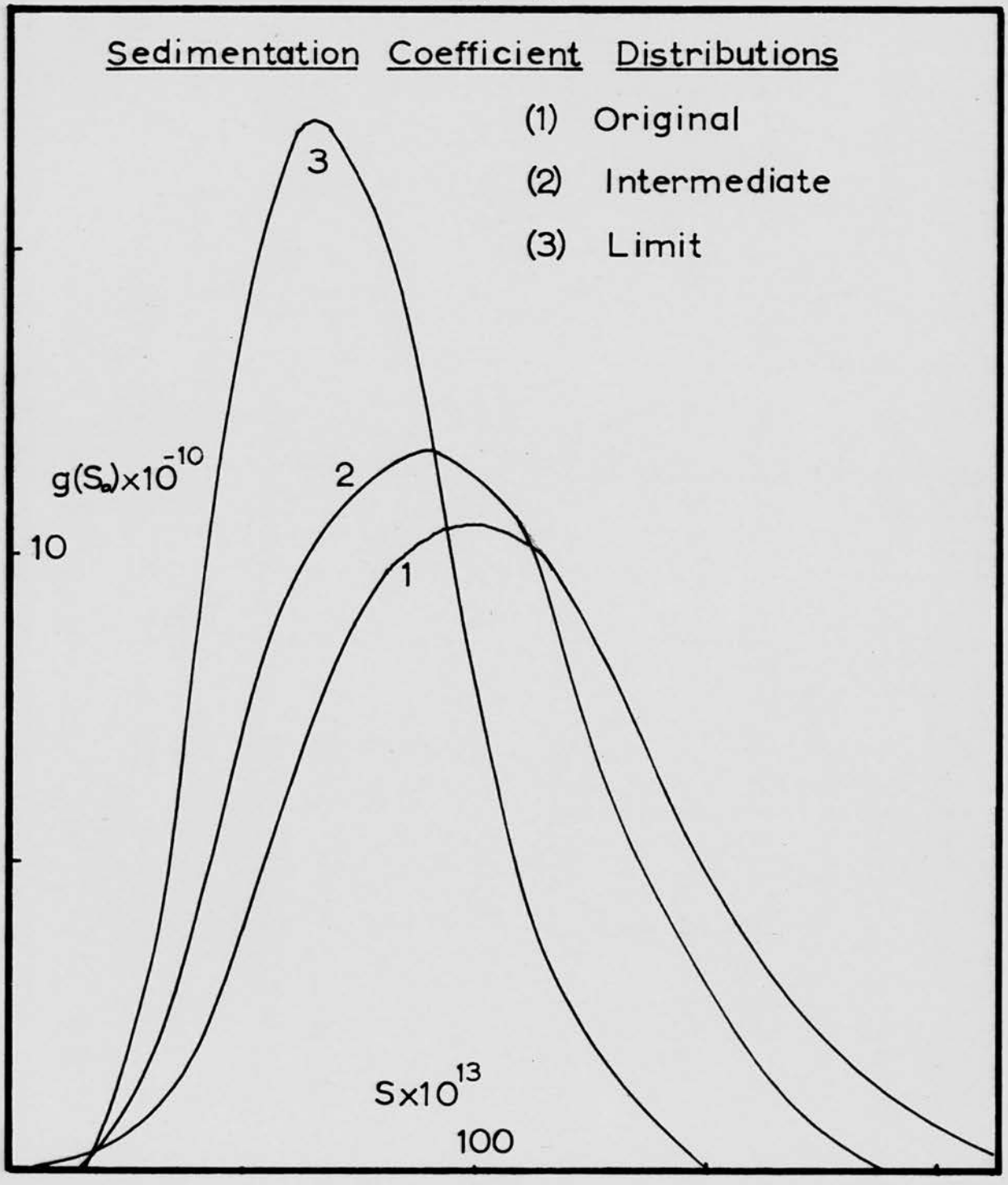
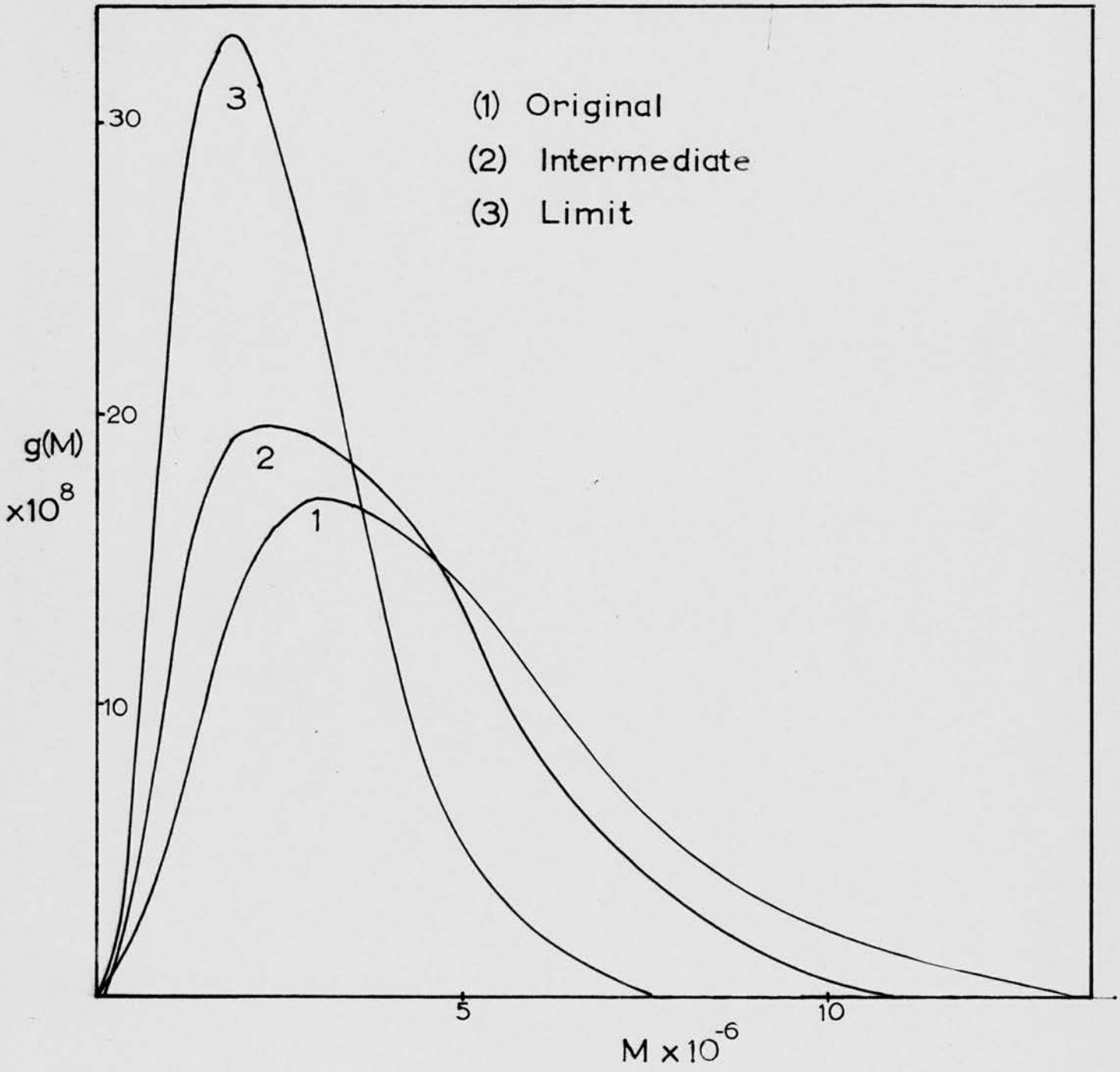

Sedimentation Coefficient Distributions
(1) Original
(2) Intermediate
(3) Limit
3
2
1
g(S)×10^-10
10
S×10^13
100

FIG. 20


(1) Original
(2) Intermediate
(3) Limit
g(M)
x10^8
30
20
10
3
2
1
5
10
M x 10^-6

RESULTS AND DISCUSSION.

Before considering how the mechanism of the degradation may be elucidated from the molecular weight distributions, the accuracy of the distributions themselves will be discussed.

Random experimental error is very difficult to assess for such a procedure. As at each stage the distribution curves were normalised, it is probable that rounding-off errors will be largely compensated for. The initial measurement of the sedimentation diagrams probably introduces the largest error, as the image on the plate is confused by interference fringes. The outline of the image of the bar is therefore not sharply defined. This diffused region around the image makes direct measurement by cathetometer unreliable, especially at the extremes of the peak. For this reason, it was found to be preferable to make photographic enlargements of the sedimentation boundary on a high-contrast paper, thereby improving the sharpness of the image. On these enlargements, the boundary could be drawn in and a base-line fitted with greater confidence. As, in most cases, the concentration calculated from the diagram agreed reasonably with the actual concentration, it is thought that the error introduced at this stage was not large.

The form of the relations of sedimentation coefficient with concentration, and with molecular weight, is a source of systematic error. The relations found to be applicable in this work differ markedly from those used by Larner, Ray and Crandall (1956) and as a result the distributions, found for what are probably very similar samples, are very different from those presented here.

Thus for concentration dependence, Larner's

$$S = S_o - 1{\cdot}6 \times 10^{-3} S^2 c^2 \qquad (1)$$

is to be compared with $S = S_o (1 - 0{\cdot}1c)$ (2)

(1) indicates very much higher concentration dependence than does (2). For example, at $S_o = 200$ and $c = 1$ gm/100 ml.

(1) gives $S = 160$

(2) gives $S = 180$

However, at lower S, the divergence is not so marked since at $S_o = 100$, $c = 1$ gm/100ml.

(1) gives $S = 88$

(2) gives $S = 90$

At this level, the difference is hardly more than experimental error. However, it is the components of large sedimentation coefficient for which this concentration dependence is most important, as these always sediment in the solution of highest concentration. This must account for a large part of the discrepancy between the present work and that of Larner. It is felt that the relation used here is more correct as it was found to hold for S values between 50 and 170. Larner, Ray and Crandall's relation, on the other hand, was derived from measurements on samples of $S < 100$ and its applicability to the larger sedimentation coefficients is doubtful.

An even greater difference exists between the relations for molecular weight.

Larner et al give $M = 569\ S^2$ (3)

compared with $M = 2{\cdot}70 \times 10^3 \times S^{1{\cdot}59}$ (4)

At low S the divergence is again not serious, as for S = 100

(3) gives $M = 5{\cdot}7 \times 10^6$

(4) gives $M = 4{\cdot}0 \times 10^6$

which can be accounted for by the unreliability of diffusion measurements. However, for S = 200

(3) gives $M = 22{\cdot}5 \times 10^6$

(4) gives $M = 12{\cdot}3 \times 10^6$

These relations must inevitably give very different molecular weight distributions. As discussed in Section III B, there is considerable difficulty in interpreting sedimentation and diffusion measurements for very polymolecular materials. Thus, although the molecular weight found probably does not truly represent any average for the complete sample, it probably is essentially correct for the most abundant component - that is, the component corresponding to the maximum of the sedimentation diagram. The relation found here has been shown to have a theoretical significance consistent with other properties of glycogen and, although probably not absolute, it is certainly of the correct form. Larner's results, on the other hand, suggest that glycogen conforms to the random coil configuration which is inconsistent with the results of viscosity measurement. Moreover, the relation was derived from measurements on three samples covering the range 3 - $5{\cdot}4 \times 10^6$ in molecular weight and its extrapolation to higher molecular weights is doubtful.

In conclusion, while these distributions are probably not absolute, it is thought that the relative values for the three samples are of the correct order.

Action-pattern of β -amylase.

It was considered that two distinct possible mechanisms could exist. These were (

(1) A completely single-chain mechanism in which no partially degraded molecules are present. Thus the intermediate dextrin would be a mixture of molecules completely degraded to dextrin, and unchanged molecules. Since for this sample the conversion to maltose was 13% compared with the 41% of the dextrin, such a mechanism would correspond to a degradation of some 32% of all the molecules by 42%.

(2) A completely random attack upon all the molecules in the system. At any given degree of overall conversion, all molecules present would be degraded to that same extent.

It is possible that either of these mechanisms might be modified by a molecular weight specificity, when presumably the larger molecules in the system would be degraded first. In addition, the degree of conversion might vary with molecular weight, with perhaps the larger molecules being degraded to a greater extent than the smaller.

To test these various possibilities, the distribution curve of the original glycogen was taken as a starting point and hypothetical

distributions for the intermediate and limit dextrins, based upon the above mechanisms, calculated from it.

A curve for the limit dextrin was first calculated, assuming that all molecules were degraded to 41%. For this, the molecular weight (M_{LD}) was calculated from

$$M_{LD} = M_o \, (100-41) \, / \, 100$$

and $g(M)_{LD}$ from

$$g(M)_{LD} = g(M)_o \; 100/(100-41)$$

This curve is shown in Fig. 21 with the experimental curve for the limit dextrin. The agreement is within experimental error, although it is possible that a slightly greater degree of conversion for the larger molecules might yield a better fit. However, this more extensive degradation need not be greater than 2 - 3%, and this amount is within the limits of experimental accuracy.

To test the first mechanism, a curve for the intermediate dextrin was calculated by combining 68% of the original distribution with 19% of the limit dextrin distribution and then multiplying the g(M) values by 100/87 to make the area comparable with the experimental distribution.

For the second case, a distribution was calculated assuming 13% degradation of all molecules.

These curves are shown, together with the experimental curve for the intermediate dextrin, in Fig. 21 . That for the second mechanism is of better all-round fit, particularly at the high

FIG. 21

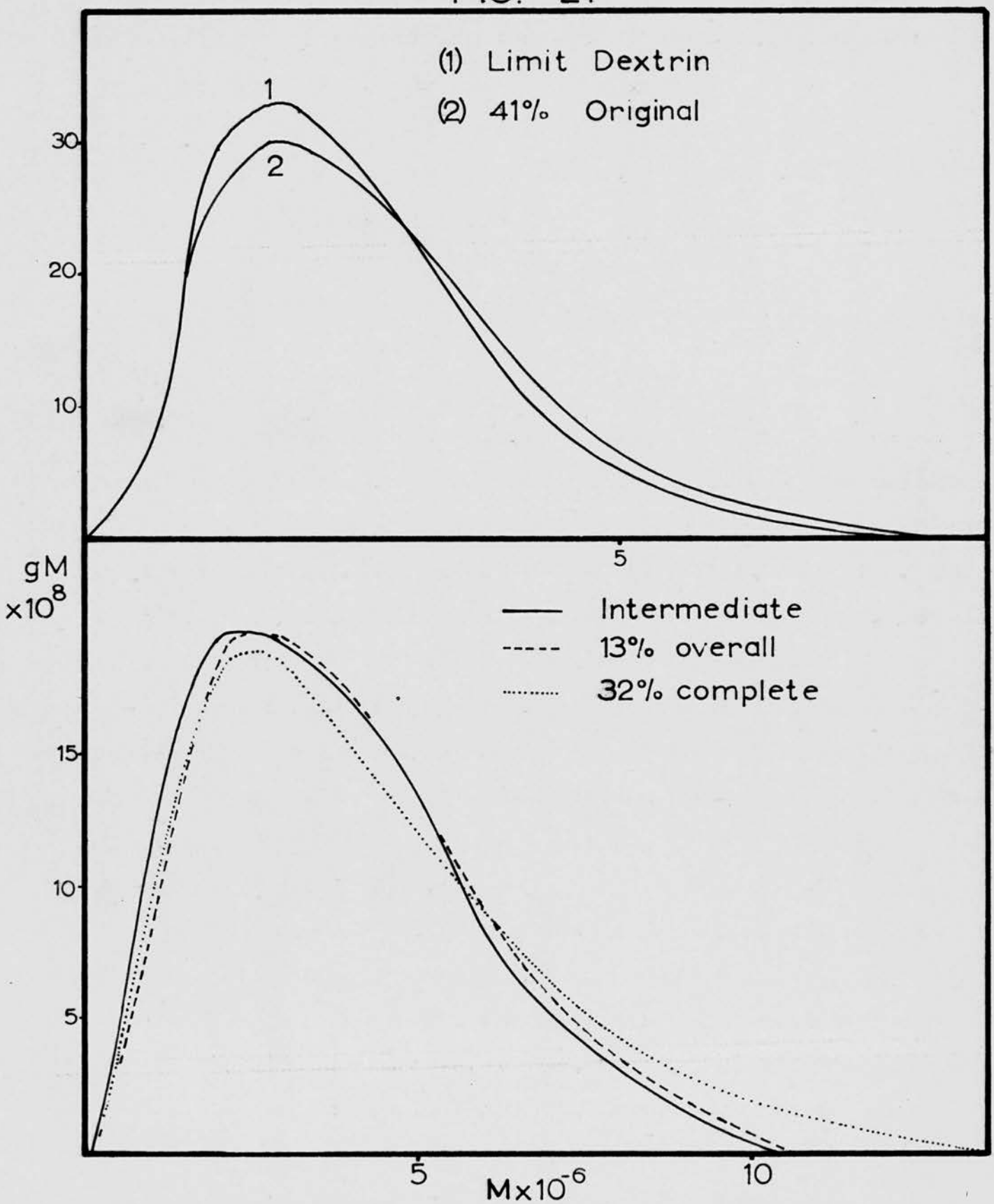
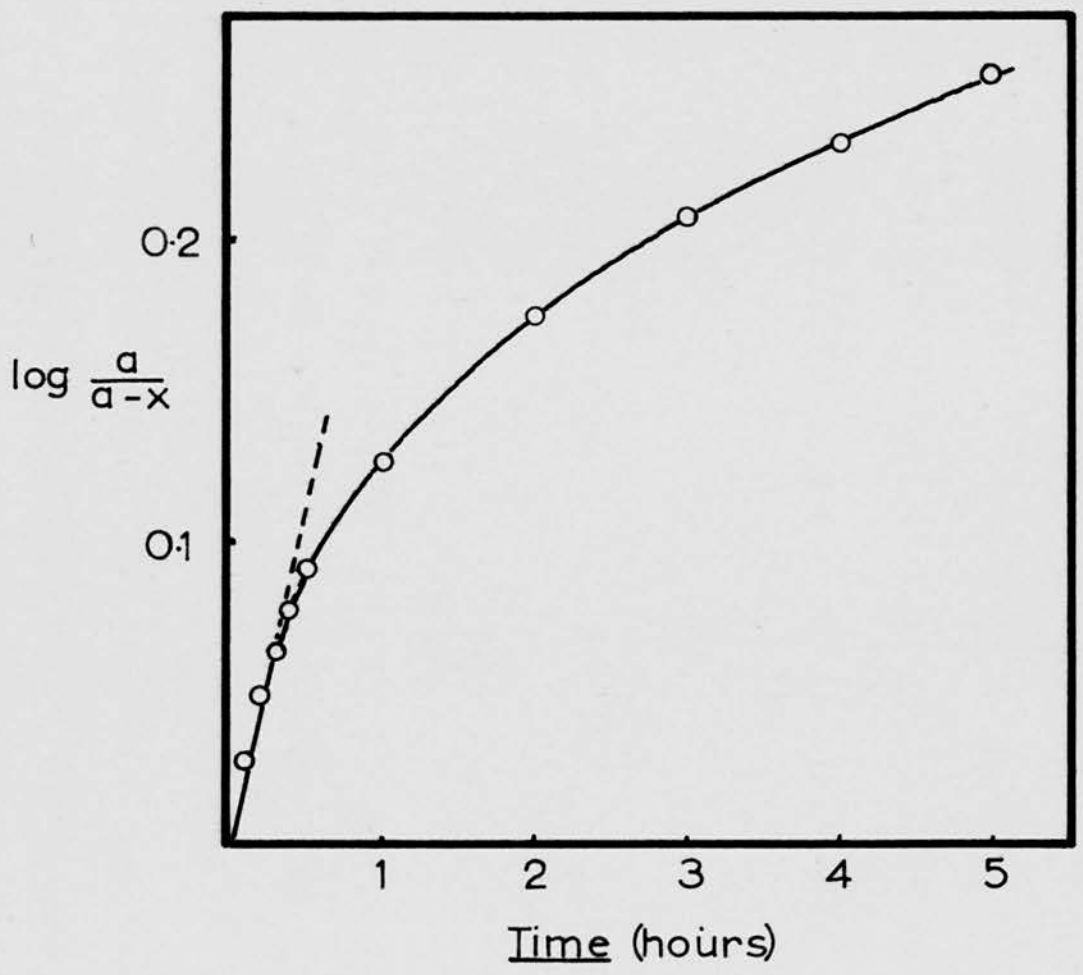

(1) Limit Dextrin
(2) 41% Original
1
2
30
20
10
5
gM
x10^8
Intermediate
13% overall
32% complete
15
10
5
5
10
M x10^-6

FIG. 22

β-Amylolysis of Glycogen



molecular weight end. The first curve has too much high molecular weight material and also, as a result, too little in the intermediate molecular weight range.

The results suggest that the degradation of glycogen by β-amylase is completely random, with all molecules being degraded to approximately the same extent..

These conclusions are supported by kinetic measurements made in conjunction with Mr. W. Banks. The rate of production of maltose was followed, and the results plotted as log $a/(a-x)$ *vs* t, where "a" is the limiting conversion and x the conversion at time t. This is the usual first order kinetic plot (Fig. 11).

The diagram shows that first order kinetics are followed for only some 10% of the total reaction, and that thereafter the rate continually decreases. When corresponding experiments were carried out on amylose and amylopectin, first order kinetics were observed over the total reaction in the first case, and over more than 80% of the reaction in the second. This suggests that successive maltose units are increasingly difficult to remove from the glycogen chains. It follows that each chain will be degraded to the same extent, consequently no molecules will be preferentially degraded.

SECTION V.

STUDIES ON POTATO AMYLOPECTIN.

INTRODUCTION.

Consistently high molecular weights have been obtained for amylopectin by many workers (Greenwood, 1956). Values for weight average molecular weights are approximately a factor of ten greater than those found for glycogen, while number averages are only slightly greater as would be expected for such highly branched polymers.

The question inevitably arises of whether these large molecular weights are real or the result of aggregation. Most determinations of weight average molecular weight have been made by light scattering and, therefore, in addition to the possibility of aggregation, it is possible that small quantities of undispersed starch granules might adversely affect the results. Both of these possibilities appear to have been ruled out by earlier work on this problem. Witnauer, Senti and Stern (1955) have shown that the molecular weight of potato amylopectin measured at 25° and 75° was the same and that, in general, light scattering measurements can be repeated within the accuracy of the method. This suggests that undispersed starch granules were not present, as it is extremely unlikely that the same contamination would be present in each measurement. Further, fractionation of the potato amylopectin yielded fractions whose molecular weights were consistent with the overall weight average. Corn amylopectin has been studied by Stacy and Foster (1956) in water, 1N KOH, ethylene-diamine and formamide and the molecular weights found were within experimental error. Erlander and French (1958) have shown that after heating

corn and waxy maize amylopectin with amyl alcohol, 0·5 N KOH and 6M LiBr - treatment which would be expected to break hydrogen bonds - high molecular weights were still obtained. The β -amylase limit dextrin of various samples has been shown by Stacy and Foster (1956) to have a molecular weight consistent with the degree of conversion to maltose by the enzyme. This suggests that aggregation through entanglement of outer chains does not occur.

It would thus appear to be well established that the high molecular weight of amylopectin is real and that aggregation is not important. However, all reliable estimates of the molecular weight of amylopectin have been made by light scattering and it was of interest to see whether a molecular weight could be derived from sedimentation measurements in view of the extremely strong concentration dependence of sedimentation coefficient which has been observed (Bryce, Cowie and Greenwood,(1957) Erlander and French,1958).

Potato amylopectin has been used in the work reported here because it can be obtained in a pure state more readily than can cereal amylopectins. This amylopectin is peculiar, however, in that it contains a small amount of phosphate. Samec (1935) has shown the phosphate to be present as an ester on carbon atom six. The presence of these ionisable groupings within the amylopectin must confer some polyelectrolyte character and the extent of this was investigated by measuring viscosity, sedimentation and light scattering of amylopectin in solutions of sodium chloride of varying concentration.

EXPERIMENTAL.

Isolation of Amylopectin.

Potato starch (var. Epicure) was dispersed at 0·5% concentration in water by boiling for one hour under nitrogen. The dispersion was allowed to cool to 60° with nitrogen passing, and then powdered thymol was added to give a saturated solution (0·1%). The flask was stoppered tightly and left for three days, during which period the thymol-amylose complex formed. This latter was then removed by centrifugation in the Sharples Supercentrifuge. The solution of amylopectin was freed from thymol by shaking with several changes of ether, and the volume reduced to about one fifth by vacuum distillation at 35° - 40°. The amylopectin was obtained as a bulky solid by freeze-drying. Potentiometric iodine titration (Anderson and Greenwood, 1955) showed that less than 1% of amylose was present.

A 1% solution of the freeze-dried amylopectin was then prepared and dialysed against running water for two days and then against three changes of distilled water over a period of two days, before finally freeze-drying. This material was used in all subsequent experiments.

Preparation of solutions.

Solutions for viscometry and sedimentation were prepared by weight from amylopectin dried in a vacuum oven at 70° overnight. Solutions in neutral solvents were made by heating on a boiling water bath for five minutes, but solution in alkali was effected by shaking in the cold.

Sedimentation measurements.

These were made in the usual manner for solutions in water, 10^{-3} M NaCl and 0·1 M NaOH. For measurements in 0·1 M NaCl the sedimentation boundary was extremely wide and diffuse and therefore it was necessary to use a synthetic-boundary-forming cell in order to obtain sedimentation coefficients of reasonable accuracy.

Light scattering measurements.

The angular distribution of scattered light was measured in water and in 0·1 M NaCl over the range 20° - 110° at 546 mμ. Solvents were clarified by filtration through G5 sintered glass. Amylopectin solutions (0·6%) were centrifuged for one hour at either 26,000 r/m or 15,000 r/m. As shown later, the higher speed caused some loss of amylopectin and therefore the lower speed was finally selected as being the more suitable. After centrifugation, the solution was decanted carefully and diluted tenfold. The dilute solution was then filtered through a G4 filter. Aliquots of the solution were added to the scattering cell to give concentrations in the range (1 - 20) x 10^{-5} g/ml.

The refractive index increment was found to be 0·150 ml/gm.

RESULTS.

The experimental results are collected in Figs. 23-28

Sedimentation: The sedimentation results showed a very strong dependence of S on c and the necessary extrapolation to zero concentration was found to be best made by plotting 1/S against c. This reduces the data to a straight line except for the alkaline solutions, but even here the curvature is much reduced and an extrapolation can be made with reasonable confidence. Fig. 23 shows how the concentration dependence is considerably reduced in sodium chloride solutions. An interesting consequence of this decrease is the increased spreading of the sedimentation boundary. In water solution, very sharp boundaries are obtained due to the strong Johnston-Ogston and boundary-sharpening effects. In salt solution these are much reduced and the shape of the boundary approaches more closely that expected for a polymer of very wide molecular weight distribution (Fig. 24). However, even in 0·1 M NaCl the concentration dependence is very large and considerable boundary sharpening must still occur. The actual molecular weight distribution must be very wide indeed.

Viscosity. Fig. 25,26 shows the effect of salt concentration upon the viscosity of amylopectin solutions. The intrinsic viscosity in 10^{-5} M NaCl is effectively the same as in pure water, but in 10^{-2} M NaCl it has fallen to one quarter of this value. At the same time, the concentration dependence of η_{sp}/c falls considerably and in the stronger salt solutions is effectively independent of concentration.

FIG. 23

(1) H_2O (3) 0·1 M NaCl

(2) 10^{-3} M NaCl (4) 0·1 M NaOH

(5) 0·1 M (NaOH + NaCl)

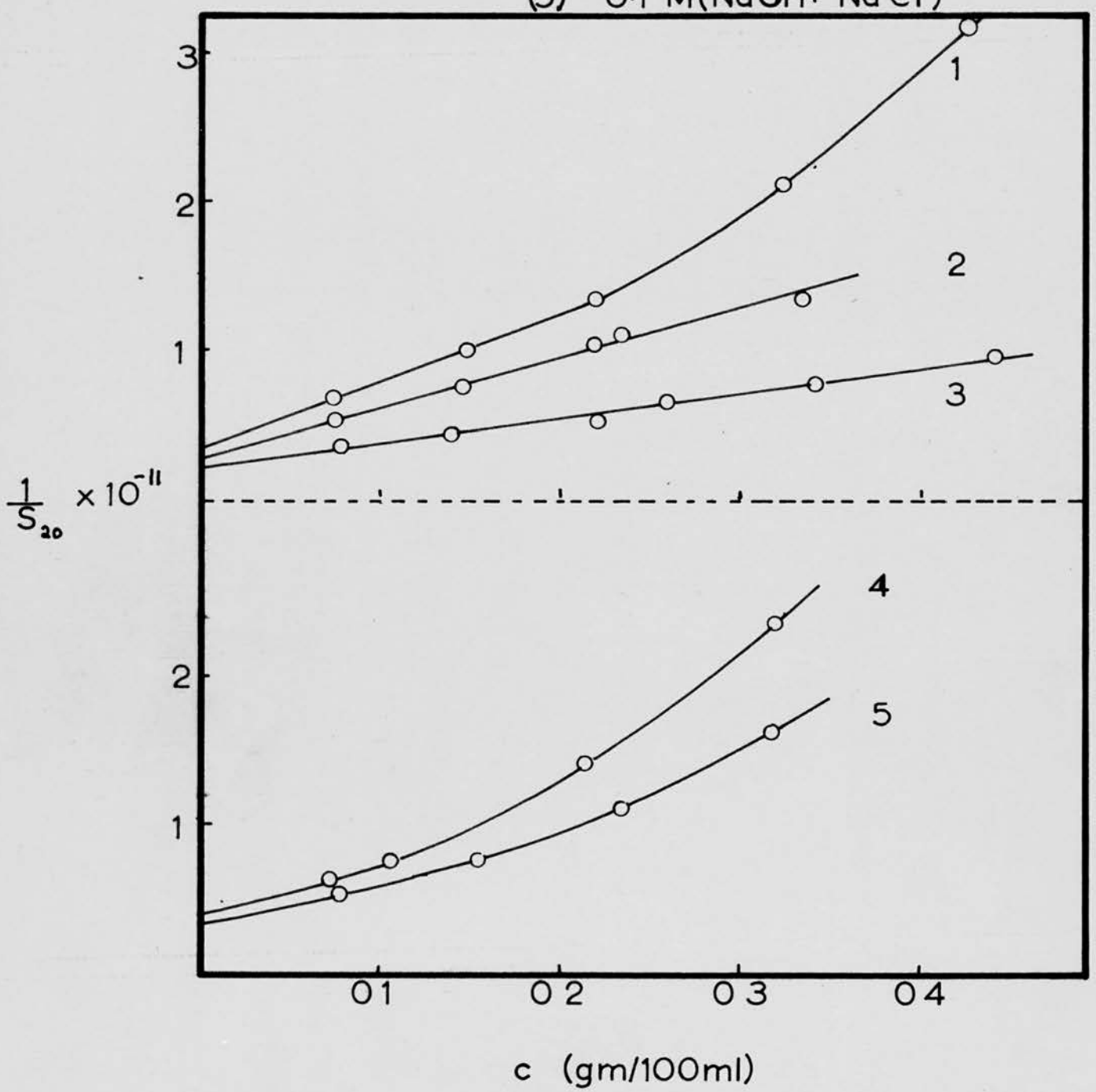


FIG. 24

Tracings of sedimentation patterns
c = 0·15 gm/100ml.
Bar angle = 55°

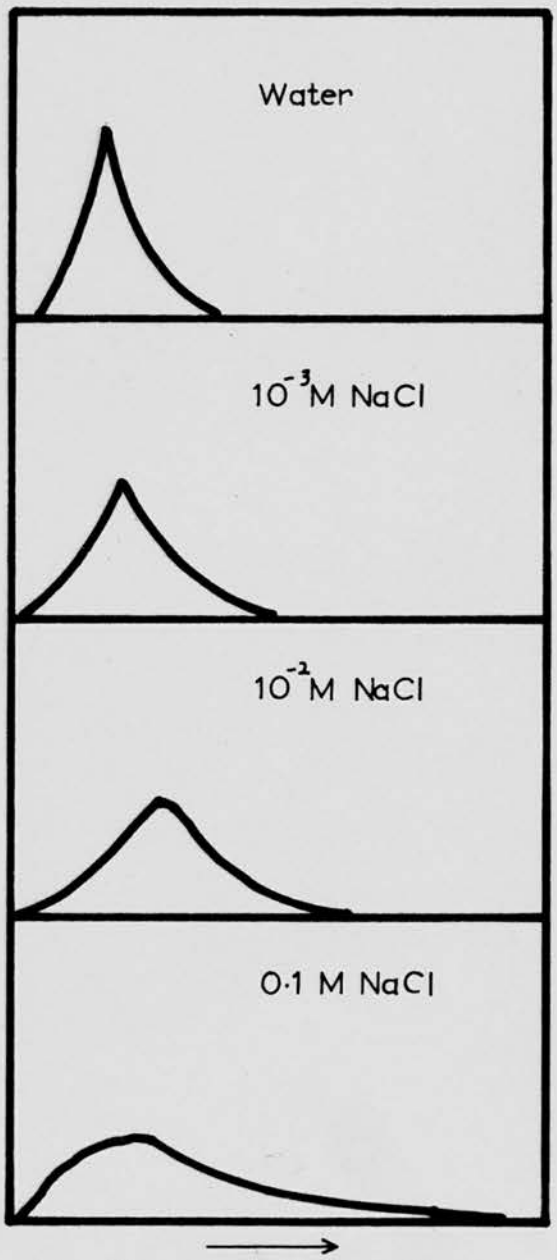


FIG. 25

$\frac{\eta_{sp.}}{c}$ vs c for Amylopectin.

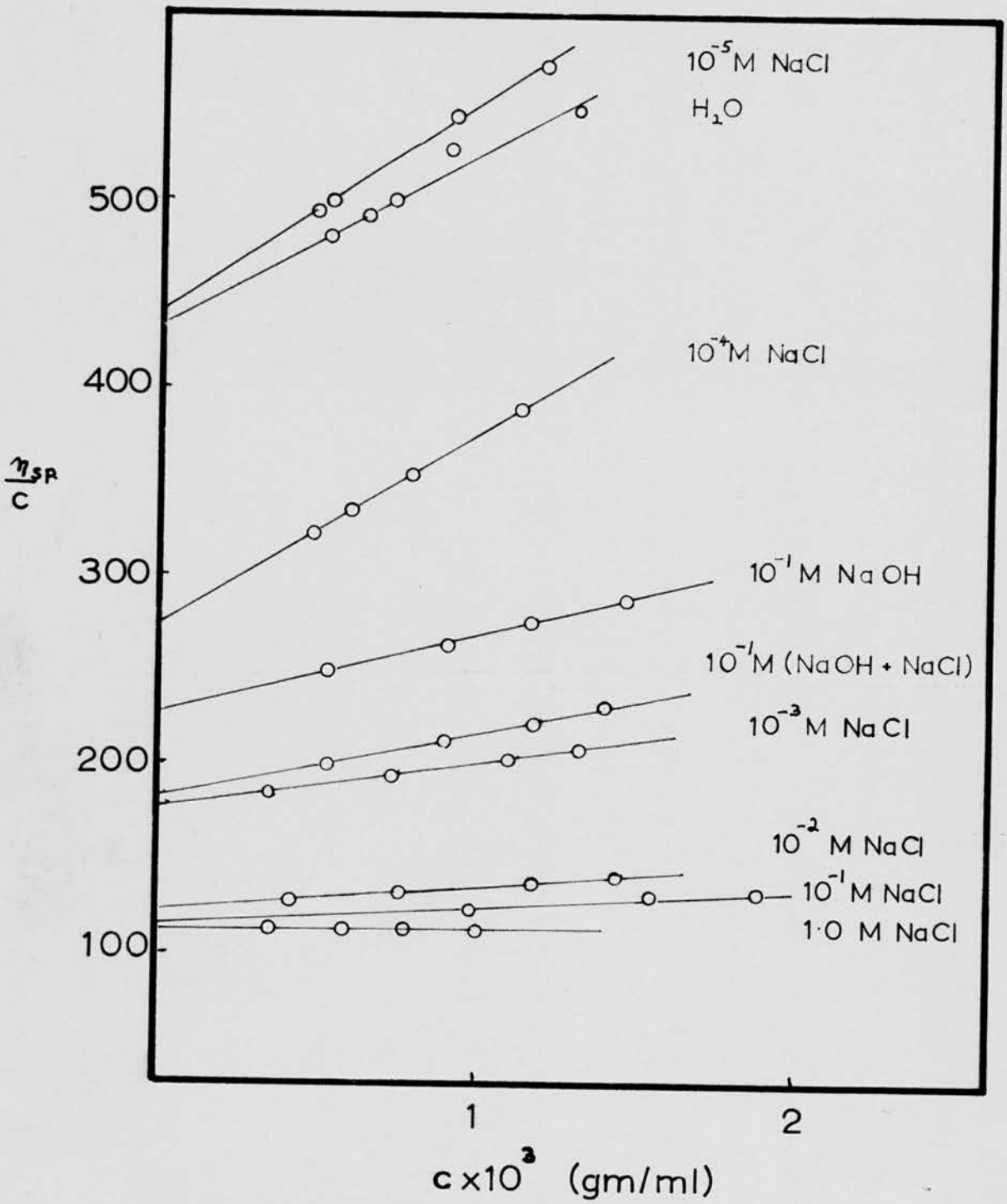


FIG. 26

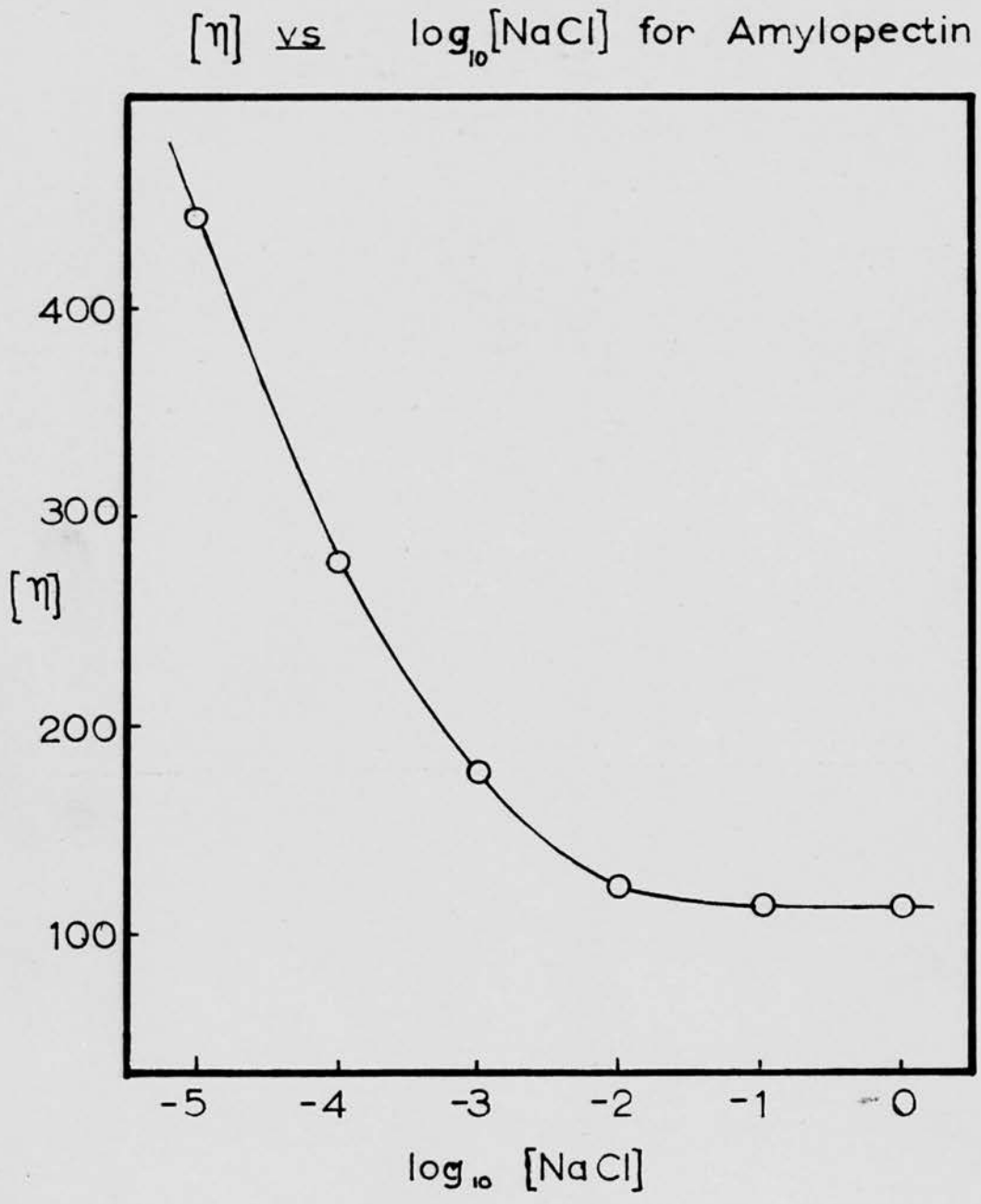

[η] vs log10[NaCl] for Amylopectin
400
300
200
100
[η]
-5
-4
-3
-2
-1
0
log10 [NaCl]

FIG. 27

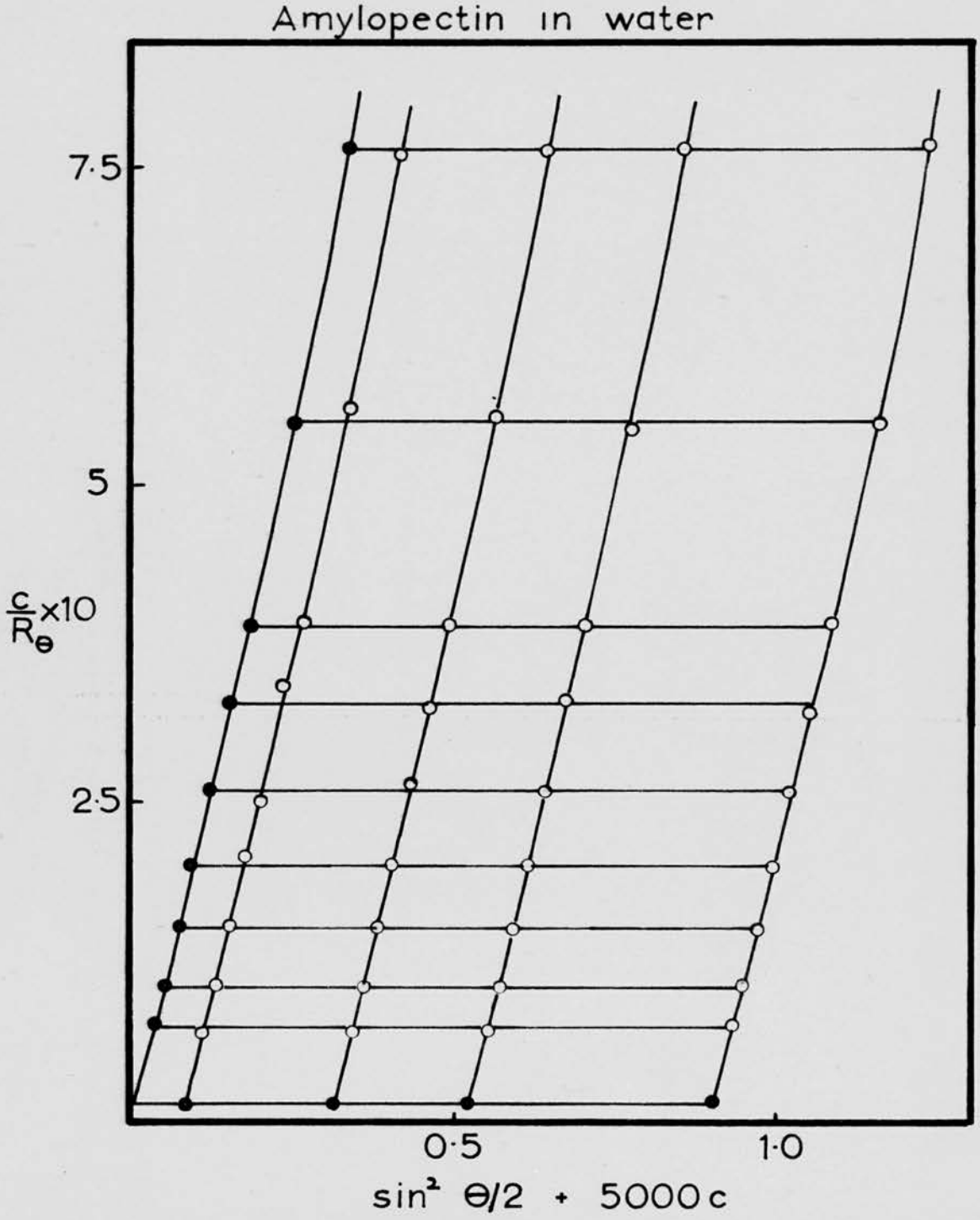
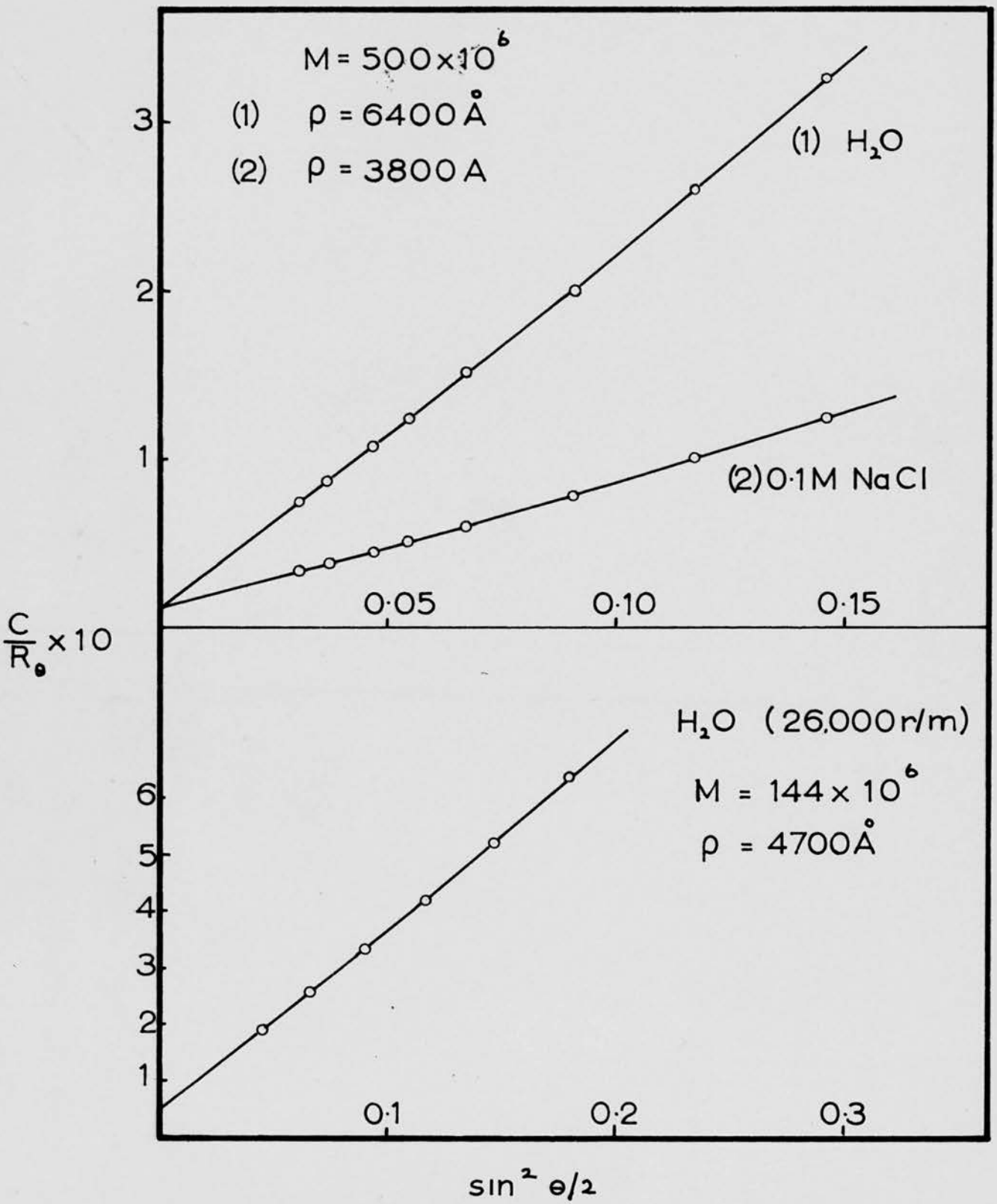

Amylopectin in water
7·5
5
2·5
$\frac{c}{R_\theta} \times 10$
0·5
1·0
$\sin^2 \theta/2 + 5000c$

FIG. 28


M = 500 x 10^6
(1) ρ = 6400 Å
(2) ρ = 3800 A
(1) H2O
(2) 0·1M NaCl
3
2
1
0·05
0·10
0·15
C/R0 x 10
H2O (26,000 r/m)
M = 144 x 10^6
ρ = 4700 Å
6
5
4
3
2
1
0·1
0·2
0·3
sin² θ/2

Light scattering: As stated earlier, clarification of amylopectin solutions was obtained by ultracentrifugation. When a speed of 26,000 r/m was used, definite sedimentation of amylopectin occurred and a small loss (5 - 10%) was observed. At 15,000 r/m no apparent sedimentation took place and no decrease in concentration. The values of the molecular weight found by the two procedures suggest that the largest molecules were preferentially lost.

A Zimm plot of the results in water is shown in Fig. 27 It is obvious that, in this concentration range ($1 - 20 \times 10^{-5}$ g/ml.) the concentration dependence is not sufficient to make any detectable difference in the value of c/R_θ at different concentrations. Therefore, the values obtained at four concentrations were averaged for each angle, and these results give effectively the zero concentration line of a Zimm plot. These lines are shown for water (spun at 26,000 and 15,000) and 0·1M NaCl (spun at 15,000) solutions (Fig. 28). The intercept on the c/R_θ axis is the same for both solutions spun at 15,000 r/m within experimental error. It is very difficult to locate such a small intercept precisely and therefore the accuracy of the molecular weight is not good. As the intercept is also necessary for calculation of the radius of gyration from the slope of the lines, the absolute accuracy here must also be low, although, since the slope itself can be determined fairly well, the relative values of the radii of gyration are probably good. There is a slight upward curvature of the lines between 27° and 20°. This was also observed by Erlander and French (1958). It may be that this curvature increases at lower angles not accessible to measurement,

and thus both the molecular weight and the radius of gyration are possibly overestimated.

DISCUSSION.

For a branched polymer, Zimm and Stockmayer (1948) have shown that

$$[\eta] = K M^{0.5} g^{3/2} \alpha^3$$

where g is a branching coefficient

α is the volume expansion factor defined by

$$\alpha = \left(\overline{\rho^2}/\overline{\rho_0^2}\right)^{1/2}$$

ρ_0 is the radius of gyration of the molecule in its most contracted form.

In the present series of measurements K, M and g are constant and therefore

$$[\eta] \propto \alpha^3$$

From the Scheraga-Mandelkern equation (p. 52) it follows that

$$S_0 \propto 1/\alpha$$

Thus changes in $[\eta]$ and S_0 must be related, through the volume expansion factor, to changes in the radius of gyration. The changes in $[\eta]$ must obviously be more marked and it is interesting to compare the ratio of α_1/α_2 from viscosity with ρ_1/ρ_2 from light scattering.

Thus for water (2) and 0·1$\bar{M}$ NaCl (1)

$$\alpha_1/\alpha_2 = \sqrt[3]{[\eta]_1/[\eta]_2}$$
$$= \sqrt[3]{115/440}$$
$$= 0{\cdot}64$$

and $\rho_1/\rho_2 = 3800/6400$
$$= 0{\cdot}59$$

These values therefore agree.

Further, the ratio $S_0(2) / S_0(1) = \alpha_1/\alpha_2$
$$= 290/470$$
$$= 0{\cdot}62$$

The good agreement here is largely fortuitous as the extrapolation of 1/S to c = o is not without error.

It is therefore obvious that the volume of the molecule is considerably less in salt solutions. This is further reflected in the reduction of concentration dependence of both sedimentation coefficient and viscosity. This confirms the supposition that concentration dependence is primarily due to molecular entanglement.

A qualitative explanation of the effect of salt can be given in two ways. In either case the primary cause must be the presence of ester phosphate groups in the amylopectin. Mr. W. Banks has shown that, for this amylopectin sample, approximately one glucose unit in two hundred is esterified with phosphoric acid. The first explanation is based upon osmotic pressure. The counter-ions associated with the phosphate group - either Na^+ or H^+ - must have a local high concentration within the amylopectin molecule. As a consequence of the difference in ion concentration between the interior of the molecule and the bulk

of the solution, solvent will tend to flow into the molecule and an expansion pressure will be set up. In the presence of a large excess of added electrolyte, no such unbalance of counter-ion concentration will exist and hence the molecule will assume a more contracted form. An alternative approach is based upon the mutual repulsion of the negatively charged phosphate groups. In pure water, the repulsive effect will be largely unhindered and the molecule is expanded. The addition of electrolyte surrounds each phosphate group with an "atmosphere" of cations and the consequent screening suppresses the repulsion and allows the molecule to assume a more contracted form (Flory, 1953).

Samec (1935) found that the first ionisation of the phosphate group was complete at about pH 3 and the second at about pH 6·5. As the present experiments were made at pH 5·2 - 5·5, the primary ionisation must have been complete and the second partly so. In 0·1M NaOH the phosphate must be completely ionised and, hence, a greater volume expansion should be produced. This is found to be the case as $[\eta]$ in 0·1M NaOH is 229 compared with 115 in 0·1M NaCl. It is possible that the additional ionisation of the phosphate is not sufficient to account for this difference. Some ionisation of the hydroxyl groups may have to be postulated. Such ionisation is quite probable as Saric and Schofield (1946) have shown that pk for the first stage of acid dissociation in starch is 13·3 and, hence, an appreciable fraction of hydroxyl groups must be ionised in 0·1M NaOH.

Molecular Weight: The light scattering results indicate that

the molecular weight of potato amylopectin is about 500 x 10^6. To obtain a molecular weight from the dynamic measurements, the sedimentation coefficient is usually combined with a diffusion coefficient. Dr. W.A.J. Bryce has found that, under his experimental conditions, the diffusion coefficient of amylopectin could not be satisfactorily measured as over a period of twenty-four hours no observable diffusion occurred. The molecular weight must then be determined from the combination of viscosity and sedimentation data in the Scheraga-Mandelkern equation (p. 52). A difficulty arises, however, over the value of β which is dependent upon the molecular shape. The molecular shape can be roughly determined by the method of Ogston (p. 114) using the concentration dependence of S. The necessary data are collected below.

Solvent	S_o	$[\eta]$	$d(1/S)/dc \times 10^{-11}$
H_2O	290	440	4·5
10^{-3} M NaCl	380	190	3·5
10^{-1} M NaCl	470	115	1·7

The axial ratios obtained by Ogston's method are J = 5 for the water solutions and $J \ll 1$ for the salt solutions. Whether the latter value indicates that amylopectin molecules are disc-like in salt solution is open to question because of the large error of the method and the unreality of the basic assumption that the molecules may be treated as rigid spheroids. However, it would appear that J must certainly be less than ten, corresponding to $\beta = 2{\cdot}4 \times 10^6$. As the lowest possible value for β is $2{\cdot}1 \times 10^6$,

an average value of $2{\cdot}25 \times 10^6$ can probably be used with little error. From the above data, the molecular weight can be calculated as 189×10^6, 187×10^6 and 198×10^6 respectively, [even if $J = 20$ is used (see later) $\beta = 2{\cdot}64 \times 10^6$ and $M \sim 160 \times 10^6$.] The agreement with the light scattering value is quite good in view of the extreme width of the molecular weight distribution (see p.138) and the many approximations made. In particular, both $\bar{M}_w$ and S_o depend upon extrapolation to a small intercept on a reciprocal scale and this is probably the greatest single source of error.

Very few previous results for potato amylopectin have appeared. Witnauer et al (1955) found 36×10^6. However, the parent starch was a commercial sample and was dispersed in air. Degradation is therefore almost inevitable and it is thought that the value of 500×10^6 is more representative of native amylopectin. Amylopectin must therefore be one of the largest molecules found in nature. It is of interest that Erlander and French (1958) have found a molecular weight of 170×10^6 for corn amylopectin and this value is more nearly comparable with the present one. These workers used much higher speeds of centrifugation ($\sim$ 60,000g.) to clarify the amylopectin solutions and hence may have caused partial fractionation as in the measurements made in the present work by centrifugation at 26,000 r/m ($\sim$ 40,000 g).

Molecular volume: The volume of the molecule can be calculated from the molecular weight and also from the light scattering radius of gyration. Comparison of the two allows an estimate of the hydrodynamic volume - V_h. This calculation has been made for water

and 0·1M NaCl.

Thus $V_h = 5000/J^2$ for water and $1100/J^2$ for 0 1M NaCl where J is the axial ratio.

When these equations are solved simultaneously with Simha's equation (p. 45) a value of $J \sim 20$ is found to be applicable. This corresponds to V_h = 12 and 3 ml/gm. respectively. These very large values of hydrodynamic volume indicate that amylopectin is a very open structure whose interior must be readily accessible to solvent. It is obviously very different from glycogen where the maximum hydrodynamic volume is less than 2 ml/gm., and, while this may indicate a fundamental structural difference, the different degrees of branching can largely explain it. Thus on the "tree" structure (p. 6), the molecular weight is given by $M = m(2^n - 1)$ where m is the weight of the chain between branch points and n is the number of tiers. For glycogen, m can be taken as about 1000 and for amylopectin, about 2000. Then for a molecular weight of 10^8 there would be 17 tiers in glycogen and 16 in amylopectin. Taking the length of a tier as 25Å in glycogen and 50Å in amylopectin - as seems reasonable from X-ray measurements (Greenwood, 1956) - the radii of the molecules would be 400Å and 800Å, if assumed to be spherical. Hence, the hydrodynamic volumes may be calculated as 1·8 and 13 ml/gm. respectively, which are close to the observed values. Thus the difference in degree of branching can satisfactorily explain the large difference in hydrodynamic volume. Of course, these results are necessarily rather inaccurate because of the many assumptions made in their

derivation and particularly because of the rather indeterminate value for the molecular shape. However, they would seem to establish, qualitatively at least, that both glycogen and amylopectin can be represented fairly well in their hydrodynamic properties by the "tree" structure.

Further work on amylopectin might be directed towards a more detailed examination of the molecular shape. This could probably be best tackled by a study of subfractions, although, in view of the disappointing results on glycogen, it is possible that this might be exceptionally difficult for such large molecules. On the other hand, the alternative approach through a study of acid hydrolysed products, is thought to introduce too many unknown variables (e.g. "weak" bonds with consequent non-random hydrolysis) to be of any value. Thus further work must be preceded by an adequate subfractionation, however difficult this may prove.

SECTION VI.

STUDIES ON AMYLOSE.

INTRODUCTION.

In investigations of the configuration of a polymer molecule in solution, it is necessary that a number of samples covering a range of molecular weight should be examined. This is particularly desirable in the case of coiled molecules where so much can be derived from the form of the relation between molecular weight and the various hydrodynamic properties. For a natural polymer, where the size of the starting material is fixed, the required range of molecular size can be obtained either by degradation or by subfractionation.

Bryce (1958) and Cowie (1958) have investigated the hydrodynamic behaviour of amylose by the use of degraded samples. While this undoubtedly yields useful results, the interpretation is difficult because of uncertainty about the change in molecular weight distribution which may accompany degradation. If the form of the distribution changes during degradation - as seems likely because of the probable presence of weak bonds in amylose (Bryce, 1959) - the relation between molecular weight and, for example, viscosity will differ for the different samples. Consequently the relation derived from the experimental data may not truly represent the behaviour of the system. On the other hand, the use of subfractionated samples gives narrower distributions of molecular weight and hence closer correspondence between the various averages of the molecular parameters. This should therefore allow more valid relations to be established.

It was therefore decided that an essential preliminary to any

further studies on amylose was the preparation of a series of adequately fractionated samples and this section describes the various subfractionation methods which were investigated. These included the standard methods of fractional precipitation and solution, and methods peculiar to amylose, based upon its ability to form complexes with various polar compounds, notably iodine and the higher alcohols.

EXPERIMENTAL.

Preparation of Amylose.

15 gm. of potato starch (var. Redskin) was dispersed in 3 l. of water by boiling under nitrogen for one hour. After cooling to approximately 60°, the dispersion was saturated with thymol and then left at room temperature for three days to allow crystallisation of the amylose-thymol complex. The complex was collected by centrifugation in the Sharples supercentrifuge and was re-dispersed in the original volume under nitrogen in boiling water saturated with butan-1-ol. The solution was allowed to cool overnight and the amylose-butanol complex collected by centrifugation. A further recrystallisation from butanol-water gave amylose of purity > 99% by potentiometric iodine titration. The amylose was either stored as the butanol complex under butanol, or was dehydrated by stirring with several changes of butanol and dried at 70° in a vacuum oven.

The intrinsic viscosity in 1M KOH was found to be 475 ml./gm., which corresponds to an approximate D.P. of 3500 (Cowie and Greenwood, 1957a).

Attempted fractionation.

(1) Fractional precipitation. A 0·5% solution of the dry amylose was prepared by boiling in water under nitrogen. It was placed in a constant temperature bath at 25° and ethanol added slowly until a precipitate appeared. This was partially redissolved by heating to 40° and then allowed to cool slowly overnight while stirring gently. The solution was then centrifuged. A very sticky precipitate was obtained. When dehydrated with ethanol, the product was almost insoluble and could only be dissolved by prolonged heating, which was found to cause extensive degradation. The precipitate itself could not be directly dissolved without similar degradation.

A similar attempt was made using a solution of amylose in ethylene-diamine. Partial precipitation with ethanol again yielded an intractable product.

(2) Fractional solution. Although the amylose precipitated by ethanol had proved so insoluble, it was thought that if the precipitate could be obtained as a thin film on an inert support, the action of solvent might be more effective. Amylose was therefore precipitated on to 0·1 mm. glass beads as described for glycogen on p. 104. Attempts to dissolve the amylose with ethanol/water mixtures succeeded in recovering only 10% of the total and, indeed, even after boiling in water for 30 minutes, some 50% still remained undissolved.

Substantially similar results were obtained on attempting to dissolve in ethanol/ethylene-diamine mixtures.

(3) Complex formation.

(a) Iodine. Rundle, Foster and Baldwin (1944) have shown that complex formation between iodine/potassium iodide and amylose in solution occurs preferentially with the largest molecules. Hollo and Szejtli (1958) have fractionated an amylose of D.P. 600 in this way. This method was tried.

100 ml. of a 1% solution of amylose in 2% Na Cl was prepared. 2 ml. of 0·01 N iodine in potassium iodide was added slowly and a blue precipitate formed. The solution was centrifuged and, to the supernatant, another 2 ml. of iodine solution was added. Sixteen additions were made in all and finally no further blue colour appeared. Each precipitate was redissolved by adding sufficient 0·01N sodium thiosulphate solution to destroy the blue colour, and immediately reprecipitating with butan-1-ol. A further recrystallisation with butanol freed the amylose from electrolyte. The viscosities of the fractions were measured and typical results are given.

	$[\eta]$
Original	370
F 1	310
F 7	370
F 15	350

Fractionation was obviously not successful and some degradation appeared to have occurred. The reason for the failure of this method probably lies in the fact that equilibrium conditions were not established as precipitation occurred immediately upon addition of iodine. It is possible that for the small amylose used by

Hollo and Szejtli (1958), precipitation was slower and more under control. However, closer examination of their results suggests that the intrinsic viscosities of the fractions are almost within experimental error and only a small amount of degradation would be required to explain them.

(b) Octyl alcohol. Lansky, Kooi and Schoch (1949) have obtained some fractionation of amylose by use of n-octyl alcohol.

A 1% solution of amylose was heated under nitrogen to 90° and 0·1 ml. n-octyl alcohol added. On cooling, a precipitate was formed. Further additions produced up to 5 fractions. These precipitates were difficultly soluble but were recrystallised with butan-1-ol. From Redskin amylose the first two fractions had $[\eta]$ = 419 and 190 respectively compared with the original 475. Here again degradation has interfered with fractionation. Examination of the results of Lansky et al showed that in all cases the average viscosity of their fractions was less than that of the parent amylose (e.g. original-275, fractions-188; original-233 fractions-176).

(c) Butan-1-ol. Goodison and Higginbotham (1950) have fractionated amylose by making use of the fact that the solubility of a butanol complex is dependent upon both the molecular weight of the amylose and the temperature. Thus amylose of large D.P. is precipitated from solution as the complex at a higher temperature than low D.P. amylose. These workers achieved fractionation by cooling a solution of amylose saturated with butan-1-ol in stages and removing the precipitate which formed by centrifugation at

at intervals of 1° between 56° and 40°. Centrifugation at constant temperature was found to be essential and this was achieved by thermostatting a Sharples supercentrifuge. The resultant fractionation was extremely good and very little degradation appeared to have occurred.

In this work, a controlled temperature centrifuge was not available and an attempt was made to obtain the same effect by filtration at constant temperature. A jacketed G1 sintered filter was made and, when packed with glass wool, it was found to remove precipitated butanol complex quantitatively from suspension.

A fractionation was carried out by cooling a 0·5% solution of amylose, saturated with butanol, slowly from 80°. When a precipitate appeared (~ 55°) the temperature was stabilised and, after stirring for one hour to allow equilibrium to be established, the suspension was filtered at the same temperature. The amylose was redissolved by passing boiling water through the filter.

On a small scale, control of precipitation was difficult to maintain and only two fractions could be obtained. However, definite fractionation had occurred.

	$[\eta]$
Original	475
F 1	516
F 2	445

When the experiment was repeated on a larger scale, the amylose was found to be more difficult to dissolve from the glass wool,

and it was necessary to remove the pad from the filter and immerse in boiling water for five minutes. Freedom from oxygen was difficult to maintain under these conditions and degradation always occurred, as in no case could a fraction larger than the original be obtained.

Conclusions.

Thus none of the methods explored yielded effective fractionation. Fractional precipitation and solution are least likely to be successful because of the poor solubility of the precipitated amylose. Those methods based upon complexing appear more promising and, in particular, the use of butan-1-ol would probably yield good results if a more efficient method of collecting the precipitated complex could be devised.

As no fractionation could be achieved it was not felt to be worthwhile to pursue any further studies of the hydrodynamic behaviour of amylose.

SUMMARY.

Current concepts in the chemistry of the starch-type polysaccharides with particular reference to fine structure have been critically discussed.

The physical methods used in this study have been outlined. In particular, the technique of light scattering has been fully discussed and a modified method of calibration, involving the use of glycogen of low molecular weight, has been developed. A new design of light scattering cell, which avoids the difficulties caused by back reflection in many other designs, has been suggested. A method for determining the molecular weight distribution of high polymers, based upon ultracentrifugal analysis, has been discussed.

Glycogens from a variety of sources have been examined in the ultracentrifuge and by light scattering. In general, the two methods yielded different molecular weights and the discrepancy was attributed to the width of the molecular weight distributions. In some cases, several distinct components were observed and their amounts and sedimentation coefficients determined.

The effect of isolation under alkaline and acid conditions upon the molecular weight of rabbit liver glycogen has been investigated and the acid-extracted material shown to have a molecular weight greater by at least a factor of ten. Differences in the sedimentation coefficient distributions for these products were determined and the significance of the molecular weight derived from sedimentation-diffusion data discussed.

The degradation of acid-extracted glycogen in boiling water has been shown to be compatible with the idea of a molecular dispersion of molecules of size-dependent stability.

Methods of subfractionation of glycogen have been critically examined. The shape of the glycogen molecule in solution has been discussed and shown to be best represented as an ellipsoid of axial ratio near six.

The degradation of glycogen by β -amylase has been followed by determination of molecular weight distribution and the mode of action of the enzyme shown to be completely random.

The hydrodynamic properties of amylopectin in sodium chloride solutions have been examined. The differences observed have been ascribed to the presence of charged phosphate groups. The molecular weight of amylopectin has been determined from sedimentation and viscosity measurements and found to be comparable with that measured by light scattering.

Several methods for subfractionation of amylose have been explored.

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Preprinted from the Journal of the Chemical Society,
February, 1958, (138), pages 711—715.

138. *Physicochemical Studies on Starches. Part XII.* The Molecular Weight of Glycogens in Aqueous Solution.*

By W. A. J. Bryce, C. T. Greenwood, I. G. Jones, and D. J. Manners.

Molecular weights are presented for 23 samples of glycogens isolated from various biological sources. Ultracentrifugal analysis showed that most of the samples were polydisperse. The molecular weights of the main components lie in the range $(3—9) \times 10^6$. The polydisperse nature of the glycogens has been confirmed by light-scattering measurements. The effects of varying the isolation procedure, and of alkali, on the molecular weight have also been studied.

Glycogen and amylopectin are both highly branched, essentially α-1 : 4-linked glucosans. However, their hydrodynamic properties are completely different. This must be related to fundamental differences in fine structure and molecular shape.[1,2] In this paper, we describe the solution properties and hydrodynamic behaviour of glycogens isolated from a variety of biological sources. Estimations of molecular weight and its distribution have been obtained, and the effects of variations in the method of isolation, and of alkali, on the molecular weight have been studied. A preliminary account of some of this work has already appeared.[3]

Experimental

Sedimentation Measurements.—The methods described in Part XI [4] were employed. M- and 0·1M-sodium chloride and 0·2M-potassium hydroxide were used as solvents.

The sedimentation constant (S_{20}) was virtually independent of the solvent, and the majority of the measurements were carried out in either M- or 0·1M-sodium chloride. Results were corrected to water at 20°.

The apparent amount of each component in a resolvable polydisperse system was estimated by direct measurement of the areas under the refractive-index gradient curves. An enlarged image (3×) of the photographic plates was projected on smooth paper and the upper outline traced. An image of the base line (from a comparative run with solvent alone in the cell) was then superimposed by alignment of the reference lines, and traced on. The refractive-index gradient curves were carefully divided, in the usual manner, on the assumption that each component had a symmetrical distribution, and the appropriate areas between the peaks and the base-line were measured with a planimeter. Values were expressed to the nearest 5%.

Estimations of the polymolecularity of the major component of some of the glycogen samples were obtained by using Gralen's function,[5] dB/dX, where B is an estimate of the "width" of the sedimentation gradient curve and is equal to H/A (A = area of the Schlieren diagram; H = the height of the maximum ordinate), and X = the distance of the peak from the axis of rotation. In all instances, B varied linearly with X. Although this function should be extrapolated to infinite dilution, the value at c = 1 g. per 100 ml. was taken as a standard for comparison of the polymolecularity of different samples.

Diffusion Measurements.—The method is outlined in Part X.[6] The solvent was 0·1M-sodium chloride, and values of the diffusion constant (D_m) were calculated by the moment method.

Partial Specific Volume.—The partial specific volume ($\bar{V}$) of glycogen was taken as 0·62, the value calculated from density measurements on aqueous solutions of one sample.

Light-scattering Measurements.—The apparatus and the methods used to clarify and dilute the glycogen solutions were similar to those previously described for the *Zea mays* polysaccharides,[6] 0·1M-sodium chloride being the solvent. Although 15% aqueous magnesium chloride has been recommended,[7,8] we found this solvent to have no advantages. The value of the molecular weight of a given sample was the same in both the above solvents. Glycogen

* Part XI, preceding paper.

solutions were clarified by careful filtration (cf. ref. 8) through sintered glass (G4). Repeated filtration caused some small loss in turbidity, whilst little improvement occurred in the apparent dissymmetry. (For example, a sample after one filtration had $M = 8{\cdot}4 \times 10^6$, dissymmetry = 1·41; after five filtrations, $M = 7{\cdot}7 \times 10^6$, dissymmetry = 1·34, the concentration being assumed to be unchanged by filtration.) Solutions were therefore filtered once, before dilution. This procedure gave reproducible results. Hc/τ was independent of c for all samples. The particle scattering factor ($P_{90°}$) was calculated from the dissymmetry, the molecules being assumed to be spherical.[6] The refractive-index increment (dn/dc) for glycogen was found to be 0·146 (c = g./ml.) in 0·1M-sodium chloride at 546 mμ.

Isolation of Glycogens.—Unless otherwise stated, samples of glycogen had been isolated from the tissue by the classical Pflüger method involving digestion with 30% aqueous potassium hydroxide at 100°, followed by precipitation of the glycogen with ethanol and with acetic acid.[9] Commercial samples of glycogen from British Drug Houses Ltd. (I), and Nutritional Biochemicals Corporation, Ohio, U.S.A. (II), were also examined. Methylated horse-muscle glycogen was kindly provided by Dr. D. J. Bell.

Results and Discussion

Sedimentation Coefficients.—Typical sedimentation data are shown in Table 1. It was apparent that for all the glycogens studied in detail, the sedimentation constant (S_{20}) was dependent on the concentration (c), and varied by about 10% for a 1% change in concentration. This is in general agreement with Larner, Ray, and Crandall's results,[10] but, whilst these authors suggested that S_{20} was a function of c^2, our values were best

Table 1. *Typical sedimentation results.*

Glycogen sample	Solvent	$10^{13}S_{20}$ at c (g./100 ml.)							
		1·0	0·75	0·50	0·25	0·16	0·125	0·08	0 (extrapol.)
Ascaris lumbricoides ...	0·1M-NaCl	47	47	47	48	—	—	—	48
Brewer's yeast	0·1M-NaCl	56	—	60	61	—	62	—	64
,, ...	1M-NaCl	54	—	—	—	—	—	—	—
,, ...	0·2M-NaOH	56	58	60	61	62	—	63	64
Commercial, II	0·1M-NaCl	65	67	69	71	—	—	—	73

represented by a linear function. The relations was expressed by $S_{20} = (S_{20})_0(1 - kc)$, where $(S_{20})_0$ is the value of S_{20} at infinite dilution, and c was expressed in g./100 ml. With the exception of the *Ascaris lumbricoides* glycogen (which was relatively concentration-independent; see Table 1), the average value of k was 0·10 ± 0·02. Values of $(S_{20})_0$ for glycogens examined at only one concentration were therefore calculated from this value, and are shown in parentheses in the second and third columns of Table 2.

Molecular Weight and its Distribution.—Table 2 shows the results of the sedimentation measurements for the 23 samples examined. Typical sedimentation diagrams are shown in the Figure. Most samples proved to be polydisperse on ultracentrifugation. Diagrams *a* and *b* (for oyster and *Helix pomatia* glycogen) illustrate the type of Schlieren diagram observed for the most obviously polydisperse samples. This feature is unusual, although Polglase, Brown, and Smith [11] reported similar results for samples of human-liver glycogen. The amounts of main components quoted in the Table are only approximate as no attempt was made to correct for boundary anomaly effects.[12] For many samples, an extremely wide molecular-weight distribution was indicated; the leading sedimentation boundary was extremely asymmetric and reached nearly to the bottom of the cell after a short time of centrifugation. It was difficult to prove whether or not this leading boundary was a second component, and hence no attempt was made to estimate either its amount or its approximate sedimentation constant. Samples which showed this probable fast component are indicated by the symbol S_{20}(F) in the Table. In some other samples, a corresponding asymmetric lower molecular weight distribution was apparent. Again, no analysis of this was attempted and this is indicated by the symbol S_{20}(S) in Table 2.

Diffusion measurements showed that for methylated horse muscle the diffusion coefficient (D_m) = $1{\cdot}0 \times 10^{-7}$; for brewer's yeast glycogen, $D_m = 1{\cdot}1 \times 10^{-7}$; for com-

mercial glycogen I, $D_m = 2{\cdot}0 \times 10^{-7}$; and for commercial glycogen II, $D_m = 1{\cdot}1 \times 10^{-7}$ The molecular weights shown in Table 2 for the main components in the other samples are calculated by assuming a value of $1{\cdot}1 \times 10^{-7}$ for D_m in agreement with other workers.[2] All the molecular weights are in the range (3—9) $\times 10^6$, and, together with the values of the frictional ratio (f/f_0), are of the same order as those previously reported from sedimentation and diffusion measurements.[2,13] It should be noted, however, that the values for

Typical sedimentation diagrams. For all samples, c = 1 g./100 ml.; solvent, 1·0M-sodium chloride; speed = 20,000 r.p.m. Movement of the peaks is from right to left. The figures in parentheses after the times indicate the angle of the Schlieren bar.

Oyster glycogen: 8 (55°), 15 (45°), 18 (35°), 30 (30°), *and* 47 (25°) *min. after reaching full speed.*

Helix pomatia *glycogen*: 6 (65°), 14 (45°), 25 (45°) 37 (45°), *and* 50 (35°) *min. after reaching full speed.*

Brewer's yeast glycogen: 9 (65°), 17 (50°), 26 (45°), 37 (40°), *and* 52 (40°) *min. after reaching full speed.*

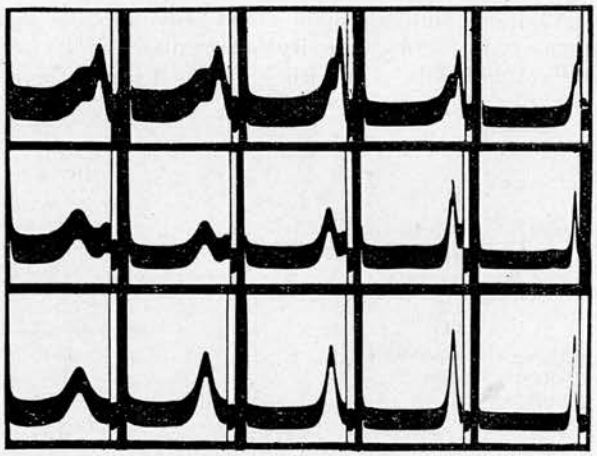

TABLE 2. *Sedimentation results.*

Glycogen sample	$10^{13}S_{20}$ of components [a] major	minor	Major component (%)	dB/dx [b]	$10^{-6}M$ [c]	f/f_0 [d]
		(a) *Mammalian livers*				
Cat I	75	F, S	—	0·8	4·4	—
,, IV	84	F, S	—	—	4·9	—
,, VI	(102)	F, S	—	1·0	5·9	—
Human (glycogen-storage disease)	(53)	(220)	70	1·8	3·1	—
Fœtal sheep	110	F	—	1·1	6·4	—
Fœtal pig	(49)	(11)	70	0·8	2·9	—
Rabbit II	94	—	95+	—	5·5	1·7
,, (fructose-infused *)	(80)	F	—	1·1	4·7	—
,, (galactose-infused *)	(153)	S	—	—	9·0	—
,, (normal *)	(145)	F	—	1·8	8·4	—
		(b) *Mammalian muscles*				
Horse (methylated)	23	—	95+	—	2·8	1·4
Human	(85)	(20)	85	0·8	4·9	—
Rabbit I	79	—	95+	0·7	4·6	1·9
		(c) *Other glycogens*				
Ascaris lumbricoides	48	F	—	—	2·8	—
Brewer's yeast	64	—	95+	—	3·7	2·0
Commercial, I	24	—	95+	—	0·7	1·9
,, II	73	—	95+	—	4·0	1·7
Helix pomatia II	(63)	(300, 7)	80	0·9	3·6	—
Mytilus edulis I	(93)	F	—	0·9	5·4	—
Oyster *	(45)	(90, F)	—	—	2·6	—
Tetrahymena pyriformis I	(69)	S	—	—	4·0	—
Trichomonas foetus I	(70)	S	—	—	4·0	—
Trichomonas gallinae I	(84)	S	—	—	4·9	—

[a] For values in parentheses and meaning of F and S, see text. [b] Value for main component at *c* (total) = 1·00 g./100 ml. [c] Molecular weight calculated from $M = \boldsymbol{R}T(S_{20})_0/(1 - \overline{V}\rho)D_m$. [d] Frictional ratio calculated from $f/f_0 = 10^{-8}[(1 - \overline{V}\rho)/D_m{}^2(S_{20})_0\overline{V}]^{1/3}$.

* Samples kindly supplied by Dr. M. Schlamowitz.

rabbit liver and muscle are considerably lower than those recently reported by Stetten, Katzen, and Stetten [8] (see below).

The values of (dB/dx) confirm qualitatively the extremely polymolecular nature of glycogen (cf. ref. 5) in agreement with the distributions evaluated by Larner and his co-workers.[10] Further, in agreement with these authors, mammalian-muscle glycogens appear to be less polymolecular than liver glycogens.

The polydisperse nature of most of the samples studied was confirmed by turbidimetric measurements. Although the molecular weight from these measurements is a true weight-average whilst that from sedimentation and diffusion is less well-defined,[14] the results from both these methods on a given polymolecular sample should be of the same order of magnitude. Polydispersity, particularly if it involves components of high molecular weight will cause disparities. Table 3 shows the results of light-scattering measurements

TABLE 3. *Light-scattering results.*

Sample	Uncorr. 10^{-6}M	Dissymmetry	$1/P_{90°}$	Corr. 10^{-6}M
Liver glycogens				
Cat I	10·5	1·48	1·30	13·6
,, IV	8·8	1·85	1·52	13·4
,, VI	12·8	1·67	1·40	17·9
Rabbit II *	6·9	1·20	1·13	7·8
Other glycogens				
Ascaris lumbricoides	7·1	1·40	1·26	8·9
Brewer's yeast *	4·0	1·15	1·10	4·4
Commercial, I	1·7	1·19	1·12	1·9
,, II *	4·9	1·15	1·10	5·4
Rabbit muscle I *	3·7	1·17	1·11	4·1
Tetrahymena pyriformis I	6·1	2·50	1·85	11·3

* Samples exhibiting no polydispersity.

on the ten samples which appeared to be the least obviously polydisperse on ultracentrifugation. For four of these, the agreement is reasonably good, indicating that they were only polymolecular, whilst the presence of S_{20}(F) in the other samples is convincingly illustrated by the higher turbidimetric molecular weight. It is therefore suggested that a given glycogen sample should be examined by both the sedimentation and the light-scattering method in order to prove unambiguously whether or not it is polydisperse. Without further investigations, it is not possible to decide whether polydispersity occurs in native glycogen in the tissue or is an artefact resulting from degradation during isolation. Polglase and his co-workers [11] consider that such variations occur naturally.

Effect of Isolation Procedure on Molecular Weight.—The classical Pflüger method involving digestion of tissue with 30% potassium hydroxide has often been criticised [15] on the assumption that alkaline degradation occurs. Table 4 shows the results for the determination of S_{20} for glycogen samples isolated from the halves of two rabbit livers severally with boiling water and 30% aqueous potassium hydroxide. Within experimental error, S_{20} is the same for all samples. It is concluded that, in the presence of air, the extent of degradation of glycogen by 30% potassium hydroxide solution at 100° is no greater than that which might be caused by boiling water. Similar results have been obtained by Staudinger,[16] and Bridgman [17] reported that glycogen extracted with cold trichloroacetic acid and hot alkali from two halves of a rabbit liver had a similar molecular weight. However, recent light-scattering work by Stetten, Katzen, and Stetten [8] has shown that if extraction with trichloroacetic acid is for a limited time at 0° the glycogen from rabbit liver has an average molecular weight of $(11—80) \times 10^6$ rather than the $(2—6) \times 10^6$ as in hot potassium hydroxide extractions. This suggests that it is difficult to avoid degradation during extraction, and that the molecular weights reported here and previously [2] may not be representative of " native " glycogen.

Effect of Dilute Alkali and Acetic Acid.—In contrast to the behaviour of hot 30% alkali,

hot *dilute* alkali appears to degrade glycogen rapidly. Digestion of rabbit-liver glycogen in 8% aqueous sodium hydroxide at 100° for 1·5 hr. reduced S_{20} from 86 to 57 $\times$ 10^{-13} c.g.s. units (see Table 4), and increased the polymolecularity (as shown by a broadening of the peak of the Schlieren pattern).

It has been suggested [18] that purification of glycogen by precipitation with glacial acetic acid may render it unsuitable for ultracentrifugal analysis. However, when rabbit-liver and brewer's yeast glycogens were reprecipitated with 80% acetic acid there was no change in the value of S_{20} (see Table 4). Precipitation of glycogen by acetic acid does not, therefore, alter the hydrodynamic properties or cause degradation of glucosidic linkages to any appreciable extent.

TABLE 4. *Effect of isolation procedure on the sedimentation constant.*

Sample	Method of isolation	$10^{13}S_{20}$ at c = 1 g./100 ml.
Rabbit liver XII	Hot water	85
	30% KOH at 100°	86
Rabbit liver XIII	Hot water	76
	30% KOH	83
	30% KOH + repptn. with AcOH	83
Rabbit liver IV	30% KOH	86
	30% KOH + 8% NaOH at 100° for 1½ hr.	57
Brewer's yeast	30% KOH	64
	30% KOH + repptn. with AcOH	63

The authors thank Professor E. L. Hirst, F.R.S., for his interest in this work, and the Rockefeller Foundation for financial support, also the D.S.I.R. for a maintenance grant (to W. A. J. B.).

DEPARTMENT OF CHEMISTRY,
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PRINTED IN GREAT BRITAIN BY RICHARD CLAY AND COMPANY, LTD.,
BUNGAY, SUFFOLK.

Preprinted from the Journal of the Chemical Society, October, 1958, (714), pages 3558—3563.

714. *Physicochemical Studies on Starches. Part XV.* The Action of β-Amylase on Glycogen as shown by Molecular-weight Distribution.*

By W. A. J. Bryce, J. M. G. Cowie, C. T. Greenwood, and I. G. Jones.

The changes in molecular-weight distribution occurring during the action of β-amylase on glycogen have been investigated by analysing the sedimentation diagrams obtained on ultracentrifugation of the original glycogen and two dextrins. The resultant distribution for the β-limit dextrin suggests that all the glycogen molecules in the sample, independently of molecular size, are hydrolysed to the same relative extent. Examination of an intermediate dextrin showed that during β-amylolysis, a mechanism involving degradation of all the polysaccharide molecules to the same extent appeared to be the most probable. The significance of these results is discussed.

Methods are available for converting sedimentation-velocity diagrams obtained on the ultracentrifugation of a polymer solution into molecular-weight distributions.[1–6] Little work of this type has been carried out on the components of starch. These studies are extremely valuable, however, as the pattern of action of any degradative agent can be followed *on a molecular basis.* As a preliminary to such studies on the starch components, we have analysed the changes in molecular-size distribution occurring during the action of β-amylase on glycogen in order to investigate the action-pattern of the enzyme and determine whether it has any degree of specificity with regard to the molecular size of the substrate. (This enzyme attacks the outer chains of glycogen and hydrolyses $45 \pm 5\%$ of the polysaccharide into maltose.) No previous work on this problem has been reported, although a similar study of phosphorylase action on glycogen has been made recently by Larner, Ray, and Crandall.[7] Glycogen has the advantage for this work that it possesses the most ideal sedimentation behaviour of all the starch-type materials; the concentration dependence of its sedimentation coefficient is small,[8] and this simplifies the calculations involved.

Our recent work [9] has shown the observed boundary gradient curve of glycogen to be very wide and dependent on the method of isolation. For an analysis of the distribution of sedimentation coefficients $g(S)$, a relatively narrow molecular fraction is preferable. This is not easily obtained from material of high molecular weight,[9] and hence a subfraction of alkali-extracted glycogen was used for these studies.

If the diffusion coefficient is negligible and the sedimentation coefficient (S) is independent of the concentration (c), the refractive-index gradient curve can be converted directly into a distribution of sedimentation coefficients $g(S)$ by the expression (cf. Bridgeman [1] and Baldwin [3]):

$$g(S) = (\mathrm{d}c/\mathrm{d}x)\omega^2 t x^3/c_0 x_0^2$$

Here, ω = angular velocity (in rads./sec.); t = time (in sec.) from the start of the sedimentation; x = distance (in cm.) of a point in the boundary from the axis of rotation; x_0 = distance (in cm.) of the meniscus from the axis of rotation; and c_0 = total concentration of the solution. However, the above conditions are obeyed by few polymers, and $g(S)$ has normally to be corrected for three boundary effects, (1) the spreading with

* Part XIV, *J.*, 1958, 2629.

time due to diffusion, (2) the anomalous apparent concentration of any individual molecular species due to heterogeneity—the Johnston–Ogston effect,[10] and (3) the narrowing due to the concentration-dependence of S. One can correct for diffusion by extrapolating an " apparent distribution " $g^*(S)$ *versus* $1/xt$ to infinite time.[2] One can correct for the other two effects either by extrapolating curves of $g^*(S)$ to infinite dilution,[4, 5, 11] or by correcting the curve of $g^*(S)$ at a single concentration for the dependence of S on c. The latter method [3] was adopted here. In view of the complex series of manipulations involved, the method is given in outline below.

Experimental

Glycogen Sample.—Glycogen was isolated from rabbit livers by extraction with hot 30% aqueous sodium hydroxide, and was purified and characterised as described elsewhere.[9] Sedimentation measurements showed that the sample was polydisperse, containing, in addition to the main component, both small and very large material (cf. ref. 9). A subfraction was obtained as follows: a 1% solution of glycogen in 0·1M-sodium chloride was centrifuged at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge) to remove very large material as gel. Cold ethanol was then slowly added to the supernatant liquid at 0°, to give a faint yet stable precipitate (approx. 30% by volume of alcohol was required). The precipitated glycogen was then removed by centrifugation at 1500 r.p.m. for 20 min. at 0°; under these conditions, the component of low molecular weight remained in solution.

Enzyme Preparation and Digest Conditions (with W. Banks).—β-Amylase was isolated from soya-beans as described by Peat, Pirt, and Whelan.[12] It contained only an insignificant trace of maltase, and no Z-enzyme as shown by experiments on potato amylose fractions.[13] α-Amylase was also absent as shown by the molecular size of the β-limit dextrin of both amylose [14] and the glycogen (see below). The activity of the enzyme in Hobson, Whelan, and Peat's units [15] was *ca.* 20,000 units/ml.

Glycogen (1 mg./ml.) was incubated with β-amylase in the presence of 0·2M-acetate buffer of pH 4·6 at 35°. Although conversion was complete within about 2 hr., the digest was left for 24 hr. before the enzyme was deactivated by heating it on a boiling-water bath for a few moments, and the glycogen-product (Found: 41% conversion into maltose) precipitated from solution with ethanol. After centrifugation, the residual β-limit dextrin was washed with alcohol and dried with ether. A polymer-product at an intermediate stage of β-amylolysis was isolated similarly (Found: 13·2% conversion into maltose).

Concentrations of glycogen were determined by hydrolysis to glucose. The latter and the amount of maltose liberated on β-amylolysis were determined by alkaline ferricyanide.[16]

Physical Measurements.—Sedimentation measurements were made as described earlier.[9, 17] The time representing the effective start of the sedimentation was obtained from the point where the curve of $\log_{10} X$ against t cut the time-axis at the value of $\log_{10} X$ for the meniscus.[17] The glycogen samples were dissolved in 0·1M-sodium chloride. Sedimentation runs for distribution analysis were carried out at 20,000 r.p.m., and a series of five photographs were taken at 6 min. intervals after the boundary had completely left the meniscus (the latter requiring about 10 min.). The concentration of glycogen was approx. 8 g./l. for these measurements.

Optical System of the Ultracentrifuge and the Measurement of the Photographic Plates.—The Spinco ultracentrifuge is equipped with a Philpot–Svensson [18] optical system, which gives the refractive index gradient curve directly. However, measurements of the height and area of the peak can be complicated by Fresnel fringes.[19] For this work, we have found an inclined bar to be more satisfactory than a wire.

When an appropriate base line has been fitted, the height (dn/dx) of at least 20 equi-spaced lamellæ at distances x_1, x_2, etc., throughout the refractive-index gradient curve has to be measured. These heights, when corrected for the angle of the inclined bar and the magnification factor of the optical system, give dn/dx values which are related to the total concentration (c) by the expression:

$$c = [\int (x/x_0)^2 \,.\, dn/dx \,.\, dx]/\Delta n$$

where x, x_0 have the values given above and Δn = specific refractive-index increment of the solute. (Trapezoidal integration is sufficiently accurate for this type of work.)

Our initial measurements were made directly from the photographic plates by using a two-dimensional travelling microscope (reading to 0·001 cm.) after the " vertical " traverse had been carefully aligned parallel to the meniscus. Heights of the two edges of the Schlieren pattern were measured. It was found most convenient to record these values and subsequent calculations directly on to a Remington–Rand printing calculator. Later measurements were made easier by printing an enlargement (9 ×) and tracing this on graph paper, corrections then being made for the additional magnification factor.

In both instances, base lines were fitted from the average of the two areas (A) under the peak and a knowledge of the concentration (c) of the solution, since

$$c = (\mathrm{A} \tan \theta / m_1 m_2 H_1 H_2 \Delta n)(x/x_0)^2$$

where θ = angle of the inclined bar in the optical system; m_1, m_2 = magnification of the cylindrical and camera lenses; H_1 = distance between the nodal point of the condensing lens and the inclined bar; H_2 = thickness of fluid column; Δn, x, x_0 are as defined above. The calculated and the actual concentrations agreed within experimental error.

Method of Determining the Molecular-weight Distribution.—(a) *The apparent distribution of sedimentation coefficients* $g^*(S)$. This function can be derived from the relations [20] $c = c_0(x_0/x)^2$ and $S = (1/\omega^2 t) \ln (x/x_0)$. It follows that $\mathrm{d}c = \mathrm{d}c_0(x_0/x)^2$ and $\mathrm{d}S = (1/\omega^2 xt)\mathrm{d}x$, and the combination of these equations gives $\mathrm{d}c_0/\mathrm{d}S = (\mathrm{d}c/\mathrm{d}x)(x/x_0)^2 \,.\, \omega^2 xt$. The curve of $\mathrm{d}c_0/\mathrm{d}S$ *versus* S is not a conventional distribution since the area under it is not unity but c_0. Normalisation of the function therefore gives the apparent distribution of sedimentation coefficients $g^*(S)$ as:

$$g^*(S) = (\mathrm{d}c_0/\mathrm{d}S)c_0^{-1} = \mathrm{d}c/\mathrm{d}x \,.\, (x/x_0)^2 \,.\, \omega^2 x t c_0^{-1}$$

This function was calculated for each sedimentation diagram for about 20 incremental values of x (*i.e.*, x_i etc.) by taking the corresponding values of $(\mathrm{d}n/\mathrm{d}x)_{x_i}$ for $(\mathrm{d}c/\mathrm{d}x)_{x_i}$ and $\Delta x \,.\, \sum_{x=0}^{x=\infty}(\mathrm{d}n/\mathrm{d}x)$ for c_0; the proportionality factors disappear in the quotient $(\mathrm{d}c/\mathrm{d}x)/c_0$. Conversion of the values of x_i into the corresponding values of sedimentation constant S_i[by $S_i = (1/\omega^2 t) \ln (x_i/x_0)$ after correction for viscosity and temperature] then enabled the graph of $g^*(S)$ versus S to be plotted for the different times of sedimentation.

(b) *Elimination of the diffusion effect.* From the graphs of $g^*(S)$ *versus* S, values of $g^*(S)$ for discrete values of $10^{13}S_i$ (*i.e.*, 10, 20, 30, etc.) were taken and plotted as $g^*(S_i)$ *versus* $1/x_i t$. A graphical extrapolation was then made to $1/x_i t = 0$ to yield values of the apparent distribution corrected for diffusion effects [$g'(S)$]. In agreement with Larner, Ray, and Crandall's results,[7] the data were best represented by straight lines, and all extrapolations were made on this basis. Our results were similar to those shown in Fig. 1 of ref. 7. The graph of $g'(S)$ *versus* S was thus obtained.

(c) *Transformation of* $g'(S)$ *into* $\mathrm{d}c/\mathrm{d}x$. Before corrections can be applied for the Johnston–Ogston effect,[10] the function $g'(S)$ *versus* S has to be transformed into $\mathrm{d}c/\mathrm{d}x$ *versus* x. The distribution equation can be re-written in this instance as:

$$\mathrm{d}c/\mathrm{d}x = g'(S) \,.\, x_0^2 c_0 / x^3 \omega^2 t$$

where t is now chosen as the average time, *i.e.*, the time in the middle of the run.[7] Values of $(\mathrm{d}c/\mathrm{d}x)_{x_i}$ were therefore calculated from corresponding values of $g'(S)$. When values of S_i were converted into x_i by the expression $x_i = x_0 \exp(S_i \omega^2 t)$, the graph of $\mathrm{d}c/\mathrm{d}x$ *versus* x was obtained.

(d) *Correction for heterogeneity.* In order to correct for heterogeneity, the distribution curve $\mathrm{d}c/\mathrm{d}x$ *versus* x is divided into a number of equi-spaced lamellæ, and these are regarded as different components. Every molecular species i present in a given plane x_j changes in concentration at that plane, if its sedimentation coefficient S_i varies with the total concentration c_i. Baldwin [3] has shown that the change in concentration of the species (Δc_i) is related to the change in its sedimentation coefficient (ΔS_i) in terms of a parameter $(r/\omega^2 x)$, where

$$r/\omega^2 x = \ln (x_j/x_0)/\omega^2 t = S_i + c_i(\Delta S_{i/} \Delta c_i)$$

whence

$$\Delta c_i = c_i \Delta S_i / \{[\ln (x_j/x_0)/\omega^2 t] - S_i\}$$

To carry out these calculations, about 20 values of $\mathrm{d}c/\mathrm{d}x$ at a fixed increment, Δx, were tabulated against x, and the parameter $\ln (x/x_0)/\omega^2 t$ was calculated. The change in con-

centration of each of the components in each successive plane was then calculated in a stepwise manner by Baldwin's method.[3]

For the first lamella (x_1), only component 1 is present and therefore its concentration $c_1 = \Delta x \,.\, (\mathrm{d}c/\mathrm{d}x)_{x_1}$.

For the second lamella (x_2), the total concentration is $\Delta x \sum_{x=0}^{x=x_2} (\mathrm{d}c/\mathrm{d}x)$, which is an increase of $\Delta x(\mathrm{d}c/\mathrm{d}x)_x$. If $S = S_0(1 - kc)$ (see p.), the sedimentation coefficient of component 1 in this lamella $(S_1)_{x_2}$ therefore decreases by an amount $(\Delta S_1)_{x_2}$ given by $-kS_{01}\Delta x(\mathrm{d}c/\mathrm{d}x)_{x_2}$ where $S_{01} = S_1/(1 - kc_1)$. From Baldwin's work,[3] the corresponding change in concentration $(\Delta c_1)_{x_2}$ is thus equal to:

$$(\Delta c_1)_{x_2} = c_1 \,.\, (\Delta S_1)_{x_2}/\{[\ln (x_2/x_0)\omega^2 t] - (S_1)_{x_2}\}$$

The true concentration of component 2 (c_{02}) is thus greater than $\Delta x(\mathrm{d}c/\mathrm{d}x)_{x_2}$ by $-(\Delta c_1)_{x_2}$.

This calculation is repeated for all the components (i) in each lamella until the corrected concentrations (c_{oi}) of each are known. Then since $c_{oi} = \Delta x(\mathrm{d}c/\mathrm{d}x)_{xi}$, it follows that $(\mathrm{d}c/\mathrm{d}x) = c_{oi}/\Delta x$. Hence values of the corrected distribution function $g(S)$ were calculated from $g(S) = (c_{oi}/\Delta x) \,.\, (x/x_0)^2 \,.\, (\omega^2 x t/c_0)$, and the graph of $g(S)$ *versus* S obtained.

(c) *Correction for the concentration dependence of S; the extrapolation of g(S) to infinite dilution.* The distribution of sedimentation coefficients at infinite dilution $g(S_0)$ is derived from $g(S) \times (\mathrm{d}S/\mathrm{d}S_0)$, since $g(S_0) = c_0^{-1} \,.\, (\mathrm{d}c_0/\mathrm{d}S_0) = c_0^{-1} \,.\, (\mathrm{d}c/\mathrm{d}S) \,.\, (\mathrm{d}S/\mathrm{d}S_0)$. Here $\mathrm{d}S/\mathrm{d}S_0$ was obtained from tabular differentiation of S_{oi} and S_i values, S_{oi} being calculated from $S_i = S_{oi}(1 - kc_i)$ where $c_i = \Delta x \sum_{x=0}^{x=x_1} (\mathrm{d}c/\mathrm{d}x)$, as above. Values of $g(S_0)$ when plotted against the corresponding values of S_0 gave the true sedimentation coefficient distribution curve.

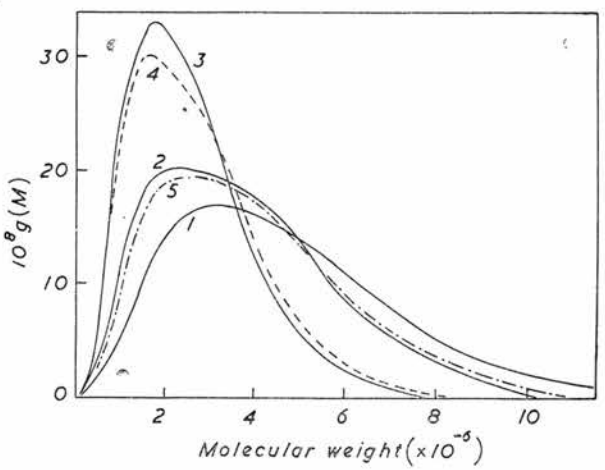
(f) *Calculation of the molecular-weight distribution curve.* Since the distribution of molecular weight $g(M)$ is given by:

$$g(M) = c_0^{-1}(\mathrm{d}c_0/\mathrm{d}M) = c_0^{-1}(\mathrm{d}c_0/\mathrm{d}S_0)(\mathrm{d}S_0/\mathrm{d}M)$$

values can be calculated from $g(S_0) \times \mathrm{d}S_0/\mathrm{d}M$. The results described elsewhere [9] enabled the value of $\mathrm{d}S_0/\mathrm{d}M$ to be obtained from differentiation of the relation (obtained by the method of least squares) between S_0 and M. This value was then utilised to calculate the curve for $g(M)$ against M.

Results and Discussion

The resultant molecular-weight distribution curves for the original glycogen (curve 1), the intermediate dextrin at 13% conversion into maltose (curve 2), and the β-limit dextrin (curve 3) are shown in the Figure. (It should be noted that these curves are weight- and



Molecular-weight distribution curves.

1, *Original glycogen.*
2, *Intermediate dextrin.*
3, *β-Limit dextrin.*
4, *Theoretical curve for β-limit dextrin (see text).*
5, *Theoretical curve for intermediate dextrin (see text).*

not number-distributions.) Although the original glycogen was subfractionated, it still possessed a very wide distribution—from about 1×10^6 to about 13×10^6—illustrating the difficulties inherent in obtaining sharp fractions of glycogen.[9]

Molecular-weight distribution curves obtained by the above methods are not absolute unless ideal polymers which are molecularly homogeneous are available: the form of the $g(S)$–S curve depends entirely on the relation between S and c, and this can be influenced by heterogeneity in molecularity. Also the transformation of the $g(S)$–S curve into the $g(M)$–M curve depends on $S = f(M)$, and this relation presents several difficulties experimentally when non-ideal samples are used: heterogeneous molecularity may influence the results, and there are often inaccuracies in measurements of the diffusion coefficients. [The method suggested by Williams and Saunders [4] for combining sedimentation-equilibrium and -velocity measurements appears to have many advantages in that diffusion measurements are avoided by using a double plot of integral distribution of M (from equilibrium measurements) and S (from velocity measurements).] For our calculations, we used the relation previously obtained [21] of $S_{20} = (S_{20})_0(1 - kc)$. Other results [9] have indicated that $dS/dM = (4{\cdot}79 \times 10^{-25})S^{0{\cdot}59}$. The latter equation is necessarily not extremely accurate, but is as good as can be expected.[9] It would appear that an *absolute* molecular-weight distribution is not yet available by this method. However, the distributions obtained here are satisfactory for comparisons on a molecular basis of various types of degradation processes. (Since Larner and his co-workers [7] used different equations, no direct comparison is possible between the shapes of his distribution curves and those reported here.)

Mode of Action of the β-Amylase.—Although an accurate estimate of the enzyme concentration in these digests was not possible,[22] conditions were such that the substrate : enzyme ratio was high. The action pattern of the enzyme was investigated by comparing theoretical distributions calculated from the original for various mechanisms with those experimentally determined for the intermediate and limit dextrin. This was achieved by dividing the distribution curve into about 20 lamellæ of discrete molecular weight, and regarding each of these as a homogeneous polymer. If the attack of the β-amylase is random with regard to molecular size, then the decrease in molecular weight during β-amylolysis will be proportional to the number of non-reducing terminal units, *i.e.*, to the molecular weight, and the molecular-weight distribution for the limit dextrin (M_{LD}) will be simply related to that of the original (M_O) by $M_{LD} = (100 - c)M_O/100$, where c = the percentage conversion into maltose (*i.e.*, 41%).

Curve 4 is the result of such a calculation. Comparison with the experimental curve (3) shows agreement within experimental error. It appears that it is essentially correct that all glycogen molecules are degraded to the same relative extent after β-amylolysis; there is no appreciable preferential and more extensive degradation of material of either low or high molecular weight.

For the intermediate dextrin, a theoretical curve was first calculated on the assumption that 32% of the molecules over the whole molecular-weight range were converted to the limit of 41% of maltose (*i.e.*, the percentage necessary to account for the observed limit of 13%), while the remainder were unchanged. However, although the maximum in the resultant distribution was correct, the high-molecular-weight leading edge was very much higher than the experimental curve, and the amount of material in the molecular-weight range of 3—6 $\times$ 10^6 was too low, the differences in each case being outside experimental error. This mechanism was therefore not compatible with the experimental results. However, when the theoretical curve (curve 5) was calculated by assuming a 13% conversion of all molecular species, good agreement was obtained with the experimental curve. This suggests that during β-amylolysis, all glycogen molecules are degraded to the same extent independently of molecular size, and the enzyme does not in fact degrade one molecule completely before attacking another. Rather it appears that action must be random with regard to individual external chains. This is in agreement with unpublished kinetic experiments carried out by Mr. W. Banks. First-order kinetics are virtually non-existent, being obeyed for only the first 10% of the total reaction, and thereafter there is a gradual decrease in rate. This suggests that as β-amylolysis progresses, it

becomes increasingly more difficult to remove successive maltose units from any chain; such a mechanism implies that essentially all molecules will be degraded to the same extent throughout the reaction.

The authors thank Professor E. L. Hirst, F.R.S., for his interest and the Rockefeller Foundation for financial support.

DEPARTMENT OF CHEMISTRY, THE UNIVERSITY,
EDINBURGH, 9.

[*Received, May 1st*, 1958.]

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PRINTED IN GREAT BRITAIN BY RICHARD CLAY AND COMPANY, LTD.,
BUNGAY, SUFFOLK.

Preprinted from the Journal of the Chemical Society,
November, 1958, (782), pages 3845—3851.

782. *Physicochemical Studies on Starches. Part XVI.* The Molecular Weight and Apparent Molecular-weight Distribution of Rabbit-liver Glycogen.*

By W. A. J. Bryce, C. T. Greenwood, and I. G. Jones.

The effects of extraction with hot alkali and cold trichloroacetic acid on the molecular weight of rabbit-liver glycogen have been examined. Molecular weights have been obtained by both sedimentation–diffusion and light-scattering measurements. Apparent distribution curves of sedimentation coefficients for various glycogen samples have been calculated. Glycogen extracted by cold trichloroacetic acid appears to be more representative of native glycogen than that isolated by hot alkali. Difficulties in the sub-fractionation of glycogen are discussed.

Our recent physicochemical studies [1] of various glycogen samples have indicated that polydispersity † is quite common, and that good agreement between the molecular weights derived from sedimentation–diffusion and light-scattering measurements is unusual. Some of the factors involved for rabbit-liver glycogen are described here, as a preliminary to use of the results as standards for the hydrodynamic behaviour of branched glucosans. The molecular weight of this glycogen from sedimentation measurements has been reported [1,2] to be about 6×10^6, but in recent light-scattering work by Stetten, Katzen, and Stetten [3] molecular weights of $11—80 \times 10^6$ were obtained when extraction was with cold trichloroacetic acid, whilst alkaline extraction gave products of molecular weights $2—6 \times 10^6$. However, determination of the molecular weight of glycogen by only one physicochemical method is inadequate.[1] In the present work, we isolated the glycogen by different methods and investigated the products by (1) sedimentation-velocity measurements, to give an apparent molecular-weight distribution, and (2) turbidimetric measurements, to give the weight-average molecular weight ($\overline{M}_w$).

For use of glycogen as a standard for hydrodynamic behaviour, fractions with a narrow molecular-weight distribution are preferable. The sub-fractionation of glycogen has therefore also been examined.

Experimental

Isolation.—Livers from freshly killed rabbits were minced and divided into two portions. The glycogen in one portion was isolated by the classical Pflüger method of extraction with 30% sodium hydroxide solution and subsequent reprecipitation with ethanol and 80% acetic acid.[1] Glycogen isolated by this method is termed *OH*-glycogen. {Typical analytical figures were: glucose, 99% (on hydrolysis and estimation of the reducing power with alkaline potassium ferricyanide [4]); $[\alpha]_D^{16}$ +194° (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 41%.} The other portion was extracted with trichloroacetic acid at 2° and the glycogen-product purified as described by Stetten, Katzen, and Stetten.[3] Glycogen isolated by this method is termed *TCA*-glycogen. {Typical analytical figures were: glucose, 98%; $[\alpha]_D^{16}$ +190 (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 45%.}

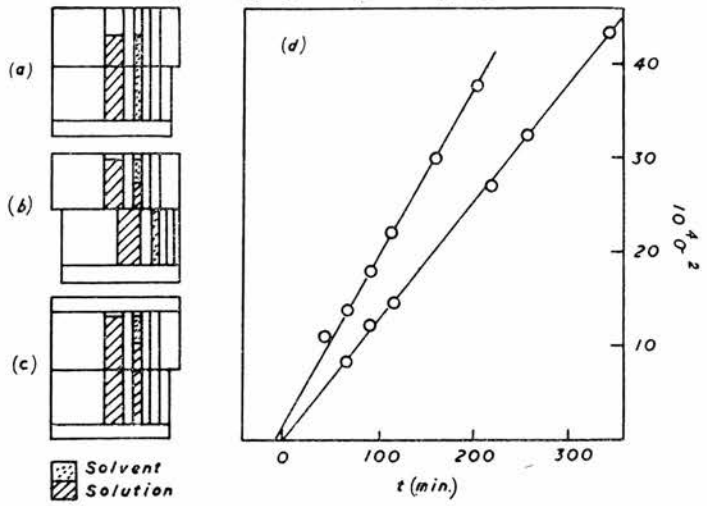
Sedimentation–Diffusion and Light-scattering.—These were carried out as described in Part XII of this series,[1] the solvent for the glycogen samples being 0·1M-sodium chloride.

* Part XV, *J.*, 1958, .

† The term "polydisperse" is used to describe a polymer system containing more than one component, whilst "polymolecular" denotes a chemically homogeneous polymer having a variation in molecular weight.

Diffusion. These measurements were carried out in the Antweiler microelectrophoresis and diffusion apparatus at 20°. The concentration at a point x in a diffusion column is a function [5] of $x/\sqrt{t}$, and hence, if x is decreased by 10, the time required to reach a given concentration is reduced by 100. The duration of the experiment can therefore be considerably decreased if dn/dx can be measured at very small values of x. In practice, as there is usually an upper limit of dn/dx which can be measured, this can be achieved only by employing a cell with a short optical path, dn/dx for given values of x and t being then proportionately decreased. Hence, with a microcell a much shorter time is required for diffusion experiments. When solvent–solution boundaries are formed in the Antweiler all-glass diffusion cell by simply sliding one compartment over the other, the position of the initial boundary is obscured and readings of refractive index gradient at this point have to be interpolated throughout the measurements. This difficulty can be avoided by filling the cell as in Fig. 1*a*. The upper compartment is then moved to the position shown in Fig. 1*b*. Careful addition of more solution to the comparison compartment (by means of a micrometer syringe) will raise the

FIG. 1. (*a*), (*b*), *and* (*c*), *Antweiler diffusion cell* (*see text*). (*d*) *Typical graphs of* σ^2 *against t for* (1) OH-*glycogen* 1, (2) TCA-*glycogen* 1.



boundary from its interfacial position. The cell is then moved into position 1*c*, after removal of the residual solvent and its replacement by solution. In this manner, extremely sharp boundaries were formed. The refractive index gradient (dn/dx) in the liquid column was obtained either by arithmetical differentiation of the results from manually scanning the column with the Jamin interferometer, or by photography of the gradient obtained directly by the Schlieren optical-system attachment. For high concentrations of glycogen, the boundaries were too sharp to be measured satisfactorily by the interference method; in other respects the results from both methods of observation were the same.

Diffusion coefficients were evaluated by either the area–maximum ordinate method (D_a) or the second-moment method (D_m), where [6]

$$D_a = \left[\int_{-\infty}^{+\infty} y \,.\, dx\right]^2 \Big/ 4\pi t(y_{\max.})^2 = A^2/4\pi t(y_{\max.})^2$$

and

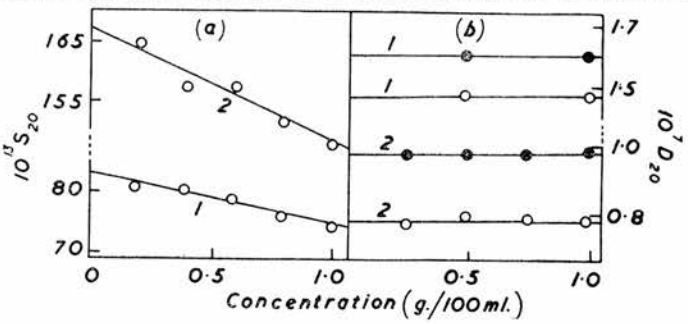
$$D_m = \left(\int_{-\infty}^{+\infty} x^2 y \,.\, dx\right) \Big/ \left(2t\int_{-\infty}^{+\infty} y \,.\, dx\right) = \sigma^2/2t$$

Here, x = the distance perpendicular to the boundary; $y = dn/dx$, the gradient of the refractive index; t = the time in seconds; σ^2 = the second moment of the curve; and A = the area. In all cases, the graph of σ^2 against t was linear and intercepted the t-axis at, or close (at a negative value) to, the origin (see Fig. 1). The time values used in calculations of diffusion coefficient were corrected for any apparent displacement of the zero time. Values are thought to be accurate to $\pm 3\%$ at any given concentration.

Ultracentrifugation. These measurements were made with a Spinco ultracentrifuge. Runs were usually carried out at 20,000 r.p.m. and at concentrations of 2·5—8·0 g./l. A speed of 8000 r.p.m. was necessary in studying the apparent molecular-weight distribution of *TCA*-glycogen. Sedimentation coefficients (S_{20}) were evaluated from measurements of the movement of the mode of the sedimentation diagram [*i.e.*, $(dn/dx)_{max.}$] in the usual manner. S_{20} values therefore represent the sedimentation coefficient of the molecular species apparently present in the largest amount.

Light-scattering. High-speed centrifugation could not be used to clarify the solutions before turbidity measurements, as rapid sedimentation of very large particles occurred (see below). Clarification was again achieved by filtration of concentrated solution through sintered glass (G4) under gravity. (Millipore filters and G5 filters were not satisfactory and tended to remove polysaccharide from solution.) For each sample, measurements were made at 4 or 5 concentrations in the range 1—10 $\times$ 10^{-5} g./ml. obtained by successive addition of the concentrated filtered solution to optically clear solvent. (The concentration of the original filtered solution was obtained by hydrolysis and estimation of the liberated glucose with

FIG. 2. (*a*) *Plot of* S_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1.
(*b*) *Plot of* D_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1; ● D_m *values*; ○ D_a *values*.



alkaline ferricyanide.[4]) This procedure gave reproducible turbidities and dissymmetries. Molecular weights were calculated from the equation: $Hc/\tau = 1/M(P_{90°}) + 2Bc/\boldsymbol{R}T$, where $H = 32\pi^3n^2(dn/dc)^2/3\lambda^4\boldsymbol{N}$; $(P_{90°})$ = a particle scattering factor, which was calculated on the assumption that the molecules were spherical; [1] B = the solute–solvent interaction parameter; dn/dc = the refractive index increment, which was taken [1] as 0·146 (c in g./ml.) for glycogen in 0·1M-sodium chloride at 546 mμ. Within experimental error, Hc/τ and I_{45}/I_{135} were found to be independent of c for the range of concentrations examined. The term $2B(P_{90°})c/\boldsymbol{R}T$ was therefore negligible.

The partial specific volume ($\bar{V}$) of glycogen was taken [1] as 0·62.

Subfractionation.—This was attempted by cooling a 0·1% solution in 15% (v/v) aqueous ethanol, and by differential centrifugation as described by Stetten, Katzen, and Stetten.[3] Stepwise addition of ethanol to aqueous solutions at room temperature was also tried.

RESULTS AND DISCUSSION

Concentration Dependence of S_{20} *and* D_{20}.—Sedimentation coefficients for rabbit-liver glycogen have been determined previously, but early investigations [7,8] were limited to only one concentration, Bridgman [7] stating that the concentration-dependence of S_{20} was less than the experimental error. However, recently we have confirmed Larner, Ray, and Crandall's results [9] that the concentration-dependence is real.[1] For the range of concentrations studied, $S_{20} = (S_{20})_0(1 - k_sc)$. Representative data are shown in Fig. 2*a*. By the method of least squares, values of k_s of 0·12 and 0·11 for *OH*- and *TCA*-glycogen respectively were obtained.

For diffusion coefficients also, early data [7,8] were restricted to one concentration. Bridgman's values [7] of $D_m = 1{\cdot}1 \times 10^{-7}$ for the majority of his samples were limiting values for time-dependent measurements. Ogston and his co-workers [8] gave values of 1·27—1·21 $\times$ 10^{-7}, whilst Larner and his co-workers' results [9] for samples of comparable

TABLE 1. *Molecular-weight data for* OH- *and* TCA-*glycogen.*

Method	Sedimentation–diffusion		Light-scattering				
Sample	$10^{13}(S_{20})_0$	$10^{-6}\,\overline{M}_{SD}$ [a]	$10^{-6}(\tau/Hc)$ [b]	I_{45}/I_{135} [b]	$10^{-6}\,\overline{M}_{\tau}$		$\overline{M}_{\tau}/\overline{M}_{SD}$
OH-Glycogen 1	84	3·1	13·6	1·70	19·0		6·1
			4·0 *	1·18 *	4·5 *		1·5
,, 2	86	3·3	—	—	—		—
,, 3 ‡	94	3·9	6·9	1·20	7·8		2·0
,, 4	95	3·9	7·1	1·20	8·0		2·1
TCA-Glycogen 1	168	9·4	38·5	2·00	62 †	}	6·3
			40·5	1·94	63 †	}	
,, 2	173	9·8	90	2·40	160 †	}	16·0
			91	2·40	162 †	}	
,, 3	163	9·1	—	—	—		—

[a] Calc. from data in Fig. 4. [b] Values at infinite dilution; I_{45}/I_{135} = dissymmetry ratio.
* Values after centrifugation at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge. Preparative rotor A.) † Independent determinations. ‡ Values from ref. 1.

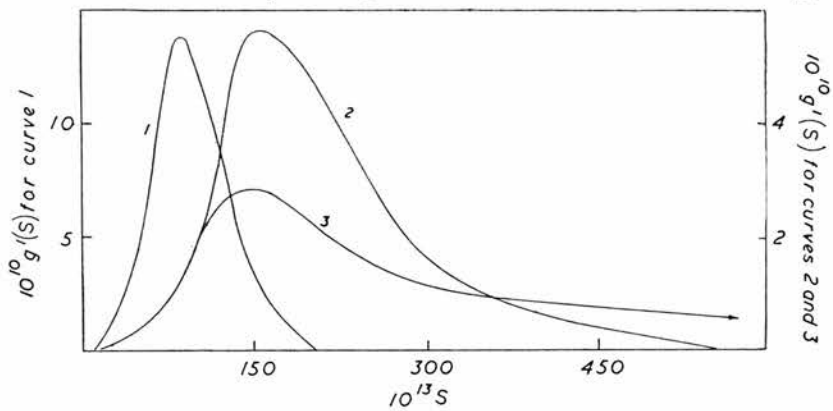
S_{20} were larger ($1{\cdot}3$—$1{\cdot}5 \times 10^{-7}$) and possessed a definite, but variable, concentration-dependence. Our experimental results (see Fig. 2*b*) suggest that the dependence is negligible. In all instances, symmetrical diffusion curves were obtained, again indicating negligible concentration-dependence.[6,10]

Comparison of OH- *and* TCA-*Glycogen with regard to Molecular Weight and its Distribution.*—The sedimentation coefficients in Table 1 indicate a large difference between *OH*- and *TCA*-glycogen. A quantitative estimate of this difference can be obtained only from the distribution of sedimentation coefficients $g(S)$, where: [7,11]

$$g(S) = (\mathrm{d}c/\mathrm{d}x)\omega^2 t x^3/c_0 x_0^2$$

where ω = angular velocity (radians/sec.), t = time (sec.) from the start of the sedimentation,

FIG. 3. *Plot of* $g'(S)$ *against* S *for* (1) OH-*glycogen* 4, (2) TCA-*glycogen* 1, (3) TCA-*glycogen* 2[$g'(S) = 0$ *at* $S = 1400$; *the amount of this sample with* S *between* 600 *and* 1400 *is about* 25%].



x = distance (cm.) of a point in the boundary from the axis of rotation, x_0 = distance (cm.) of the meniscus from the axis of rotation, and c_0 = total concentration of the solution. An absolute distribution results only if diffusion is negligible and S is independent of c. Corrections for these effects can be made.[11,12] Here, Baldwin's method [11] has been employed to correct for diffusion and obtain apparent distributions $g'(S)$ (the calculations necessary for this distribution are detailed in Part XV). If $g'(S)$ is obtained at identical concentrations and the sedimentation behaviour of the samples is the same, the resultant curves should be comparable, although corrections for the Johnston–Ogston effect [11,13] and the concentration dependence [11] of S should ideally be applied. The $g'(S)$ curves shown in Fig. 3 emphasise the radical difference between *OH*- and *TCA*-glycogen (*e.g.*, only 8% of *OH*-glycogen 4 has $S > 150$, whilst *TCA*-glycogen 1 has 61% and *TCA*-glycogen 2 has 72%). Table 2 shows calculated values of the standard deviation,

mean (or weight-average) sedimentation coefficient, and skewness. The standard deviation for *OH*-glycogen 4 calculated from Baldwin's most recent work,[14] taking into account the concentration dependence of S, is the same (*i.e.*, 36*S*) as that calculated from the $g'(S)$ curve at 8·0 g./l. Both *TCA*-glycogens have a large positive skew; that for *OH*-glycogen is relatively small. The ratio [15] D_m/D_a (Table 2) from diffusion measurements indicated the increased polymolecularity of *TCA*-glycogen.

When molecular weights were calculated from S_{20} and D_m (to give $\overline{M}_{SD}$) for four samples, then $S_{20} \approx \overline{M}_{SD}^{0.63}$ (see Fig. 4). From Kuhn and Kuhn's results,[16] (1) $S \approx M^{\frac{1}{2}}$ for a matted coil, and (2) $S \approx M^{\frac{2}{3}}$ for a sphere, and hence glycogen may behave as essentially

TABLE 2. *Sedimentation coefficients and derived quantities from $g'(S)$ curves.*

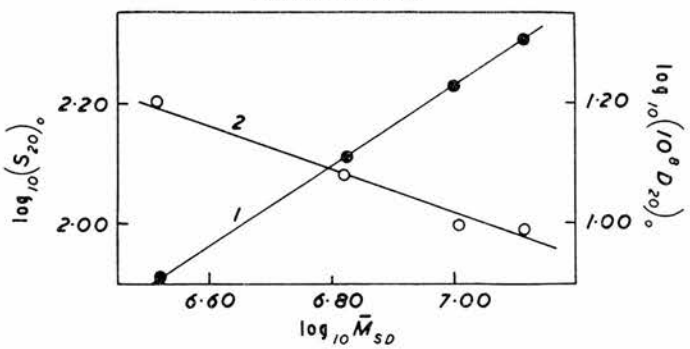
Sample	$10^{13}S_{20}$ [a]	$10^{13}S_m$ [b]	σ [c]	Sk [d]	D_m/D_a	$10^{-6}\overline{M}_{SD}$ [e]	$10^{-6}M$ [f]
OH-Glycogen 4	87	95	34	+0·23	1·08	3·9	5·0
TCA-Glycogen 1 ...	150	220	99	+0·70	1·27	9·4	18
,, 2 ...	150	417	310	+0·87	—	9·8	45

[a] Sedimentation coefficient as measured from the mode. [b] Calc. mean sedimentation coefficient [c] Standard deviation. [d] Skewness = (mean − mode)/σ (see, *e.g.*, Yule and Kendall, "Introduction to the Theory of Statistics," Griffin, London, 1950. [e] Values from Table 1. [f] Molecular weight calc. from S_m by assuming $k_s = 0{\cdot}12$ and data in Fig. 4.

spherical particles. Similar conclusions can be drawn from the fact that $D_{20} \approx \overline{M}_{SD}^{0.37}$ (see Fig. 4), an exponent of 0·33 being expected for a sphere. It is realised these relations are, at best, only approximate, as fractionated samples should have been used: this was not possible (see below). {The compact nature of the particles is confirmed by viscosity measurements. $[\eta]$ was 6·2 for *OH*-glycogen 1 and 7·1 for *TCA*-glycogen 1. (Units of concentration = g./ml.; solvent = 0·1M-sodium chloride; temperature = 22·5°.)}

Molecular weights from sedimentation–diffusion measurements on extremely polymolecular polymers are not simple averages, but depend [17] on the shape of the molecule

FIG. 4. *Plots of* (1) $\log_{10}(S_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$, *and* (2) $\log_{10}(D)_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$ *for glycogen samples.*



and the methods of evaluating S and D, and hence no direct correlation is to be expected with the results of light-scattering measurements ($\overline{M}_\tau$ values) shown in Table 1.

$\overline{M}_\tau$ for *OH*-glycogen is much less than that for *TCA*-glycogen. Differences between $\overline{M}_\tau$ for *TCA*-glycogens 1 and 2 are explained by the differences in skewness of the $g'(S)$ curves. The ratio $\overline{M}_\tau/\overline{M}_{SD}$ appears to give a qualitative measure of skewness of the distribution and values are given in Table 1. Molecular weights calculated by using mean sedimentation coefficients from the $g'(S)$ curve are more comparable with $\overline{M}_\tau$ values (see Table 2).

Stability of TCA-*Glycogen.*—The effect of various reagents on the sedimentation behaviour of *TCA*-glycogen was examined to investigate whether physical aggregation was occurring. Ultrasonic experiments (which will be described in detail elsewhere)

showed that under conditions which rapidly degrade amylopectin no appreciable change occurred in either S_{20} or the appearance of the leading edge of the sedimenting boundary. *TCA*-Glycogen also appeared to be stable to dilute acid and alkali at room temperature. After 72 hr., 0·5% solutions in 0·2M-potassium hydroxide and -acetic acid had the same S_{20} value as a control solution in 0·2M-sodium chloride, and there was no apparent change in the leading edge. When an aqueous solution (under air) was heated on a boiling-water bath, S_{20} was virtually the same after 1 hr. (158*S* ⟶ 151*S*), and even after 4 hours' heating, there was relatively little effect (131*S*). Changes in the leading edge were then apparent which did not appear to be reversible. Limited degradation or disaggregation must therefore have occurred.

The above experiments suggest that *TCA*-glycogen dissolves to form an essentially molecular dispersion. Further, $\overline{M}_\tau$ for the limit dextrin produced by the action of β-amylase had decreased by 50% compared with the 45% enzymic conversion into maltose. This is in agreement with Stetten, Katzen, and Stetten's results,[3] and suggests that aggregation was limited, as it appears unlikely that the extent of any aggregation, persisting after the 41% loss of weight on β-amylolysis, would be equivalent to that before treatment with enzyme.

However, when a 0·2% solution in 30% aqueous potassium hydroxide (under air) was heated on a boiling-water bath, S_{20} decreased rapidly and then remained constant (168*S* ⟶ 100*S* in ½ hr. ⟶ 83*S* in 1 hr. ⟶ 86*S* in 4 hr.). The sedimentation diagram was then indistinguishable from that of *OH*-glycogen. A similar effect was found when heating was in a nitrogen atmosphere. *TCA*-Glycogen appears to be alkali-labile, but oxidative degradation appears to be insignificant. *OH*-Glycogen must be a degradation product relatively stable to alkali.

TABLE 3. *Sub-fractionation of* TCA-*glycogen.*

Sample:	1			2			
Method:	Cooling to 0° and centrifugation			Differential centrifugation			
Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$	Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$
45	10·3	66	6·4	38	—	250	—
30	7·0	55	7·9	42	12·7	90	7·1
20	8·6	50	5·8	16	8·6	13·3	1·5

* Calc. from $(S_{20})_0$ value and data in Fig. 4.

Effect of Isolation Procedure on Molecular Weight.—We support Stetten, Katzen, and Stetten's conclusions [3] that *TCA*-glycogen is more representative of native glycogen than *OH*-glycogen. Extraction with hot 30% potassium hydroxide solution causes obvious degradation, and molecular-weight values reported previously [1,2] for glycogens isolated from tissues by this method are undoubtedly those of degraded products. Since degradation may also have occurred during the isolation of *TCA*-glycogen,[3] the extremely high weight-average molecular weight of this material suggests that " native " glycogen may well *not be amenable to study by conventional physicochemical methods* (compare, for example, ref. 18). The difficulties involved in the study of the size of " native " glycogen are obviously very great. It should be noted, however, that for bacteria [19] and yeasts [20] *TCA*-glycogens are smaller than *OH*-glycogens, probably because the acid has only limited access and only material of low molecular weight is extracted without prior alkaline cytolysis.

The polydispersity of *OH*-glycogens apparent on sedimentation measurements varied; some were monodisperse, whilst others had both a large and a small component. Any large component could be removed by centrifugation, no significant amount (<5%) of material being lost (see sample 1, Table 1), and reprecipitation often removed the smaller component. In view of results with trichloroacetic acid, we regard polydispersity in *OH*-glycogen as due to an artefact. It is of interest that Bridgman [7] found evidence of components of low molecular weight in some of his samples.

Our previous results [1] indicated that S_{20} for *OH*-glycogen was comparable with that for glycogen isolated by boiling water. In view of the degradative effect of alkali, glycogen in the tissues which is accessible to the solvent action of hot water must be comparable in size with the degraded product.[21]

Sub-fractionation of TCA-*Glycogen.*—The results of our experiments are shown in Table 3. No significant fractionation occurred with successive addition of alcohol. The methods suggested by Stetten, Katzen, and Stetten [3] gave limited sub-fractionation, but changes in $\bar{M}_\tau$ are due almost entirely to changes in very large material. In no instance was there any real narrowing of the apparent molecular-weight distribution as shown by the sedimentation diagrams. Sub-fractionation of *TCA*-glycogen is obviously very difficult in view of the large molecular sizes involved.

[*Added, September 8th,* 1958.—Stetten *et al.*[22] have recently reached essentially the same conclusions as ours concerning *TCA*-glycogen.]

The authors thank Professor E. L. Hirst, F.R.S., for his interest, the Referees for valuable criticism, and the Rockefeller Foundation for financial support.

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BUNGAY, SUFFOLK.